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(54) Title: METHODS FOR INCREASING THE MATURATION OF CELLS

(57) Abstract

The present invention provides a method of increasing the maturation rate or proliferation rate of a cell utilizing microencapsulation techniques. The invention also provides a method of treatment of a subject having diabetes utilizing cells produced by the culture method described herein.

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METHODS FOR INCREASING THE MATURATION OF CELLS

FIELD OF THE INVENTION

The present invention relates generally to maturating cells, in particular, neonatal islet cells. These cells are useful for transplantation, for example, into a subject suffering from a diabetic condition. These transplanted cells supplement or replace the diabetic subject's deficient pancreatic islet cells, allowing the patient to produce insulin in response to glucose without insulin injections.

BACKGROUND OF THE INVENTION

Insulin-dependent diabetes mellitus (Type I) is caused by the progressive destruction of the insulin-producing pancreatic islet cells, which eventually leads to life-long dependence by the diabetic subject or patient on insulin therapy. A major focus of diabetes research has been to develop a better treatment to correct or alleviate the symptoms of diabetes, thereby preventing the disabling complications of the disease.

One approach is to transplant isolated insulin-producing pancreatic islet cells into the diabetic subject. Clinical trials using certain types of islet transplantation have corrected the abnormally-high blood glucose levels in Type I diabetics, rendering the transplant recipients partially or totally independent of exogenous insulin therapy. For the patients which are now independent of the insulin therapy, the transplantation has restored euglycemia. Several cases have been reported wherein human islet allotransplantation has corrected basal hyperglycemia, rendering the recipients insulin-independent for varying periods of time (1-6). This recent success is attributable to the development of reproducible methods for isolating and purifying human islets (7-9) and the production of an adequate cell mass to achieve insulin-independence. If islet transplantation is to become a widespread treatment for Type I diabetics, however, the supply of donor organs must be increased.

To overcome this supply problem, islet tissue from abundant and accessible animal sources is being considered for xenotransplantation (10-17). Pigs meet the necessary requirements for a xenogeneic source of insulin-producing tissue as they breed rapidly, have large litters, and exhibit morphological and physiological characteristics comparable to humans. Porcine insulin is structurally similar to human insulin and has been safely used for treating Type I human diabetics via exogenous insulin treatment.

Unfortunately, despite many reports on the isolation of adult porcine islets, factors, such as age, breed, and quality of organs, adversely affect the final yield (18, 19). Adult porcine islets are fragile, difficult to isolate, difficult to maintain in tissue culture (17, 20, 21) and difficult to cryopreserve. The fragility of these adult islets also significantly decreases the yields of islet cells particularly when culture procedures are used to reduce graft immunogenicity or when low temperature storage is used to combine isolates from multiple donors. In contrast, tissue culture of a collagenase-digested fetal porcine pancreas produces viable islet-like cell clusters (10-12, 14), which have the ability to cure diabetic nude mice within two months posttransplantation (11, 12). However, generally, rat (22-25), porcine (11, 12), and human (26) fetal pancreatic cells exhibit a poor insulin secretory response to glucose (22-26), and the onset and maturation of glucose-induced insulin secretion is more evident in the postnatal period (22-25).

SUMMARY OF THE INVENTION

The present invention provides a method for increasing the rate of proliferation of a cell comprising encapsulating the cell within a stabilizing matrix and culturing the resulting cell *in vitro*. Also, the rate of maturation of an undifferentiated cell can be increased via the encapsulation of that cell. Preferably, the cell can be encapsulated within a microcapsule to form a microencapsulated cell. Typically, the microcapsule comprises alginate or agarose, which forms a thin layer of material around the encapsulated cell. In one aspect of the invention, more than one cell type is encapsulated within the microcapsule.

Brief Description of the Drawings

Figure 1 shows an electron micrograph of porcine NIC aggregates after nine days culture. Beta cells appear well granulated, structurally intact and contain secretory granules which conform to adult granule morphology. The aggregate also contains non-granulated cells (*) and numerous duct-like structures (arrows) (x130).

Figure 2 shows light micrographs of native neonatal (1-3 day old) porcine pancreas (A,B x250), 9 day cultured NIC aggregates (C,D x250), and porcine NIC aggregates 14 weeks after transplantation beneath the kidney capsule of alloxan diabetic nude mice (E,F x125; g,h x250). Sections were counterstained with Harris's hemotoxylin then immunohisochmically stained for insulin (A,C,E,G) or glucagon (B,D,F,H).

Figure 3 shows blood glucose values during oral (A; OGTT) and intraperitoneal (B; IPGTT) administration of glucose to nude mice transplanted with 1000 (■ ; n=10) or 2000 (▲ ; n=12) porcine NIC aggregates in comparison to age-matched normal control mice (•; n=9). *P<0.05, †P<0.01 vs. normal controls; ‡P<0.05 vs. 1000 aggregate recipients.

Figure 4 shows electron microscopy of porcine NIC aggregates 14 weeks after transplantation beneath the kidney capsule of alloxan diabetic nude mice. β cells are structurally intact, highly granulated and contain well developed endoplasmic reticulum; some cells are shown to express mitotic figures (arrows, x 6200).

DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides an *in vitro* method for enriching a population of preferred cells from a tissue source including treating the tissue source to form a preparation and then culturing the preparation *in vitro* with a serum-free basal media supplemented with a serum substitute, an agent to stimulate DNA synthesis, and an agent to promote survival of the selected cell type, thereby resulting in an enriched population of desired cells. The selected cell type can include, but is not limited to, differentiated endocrine cells, such as neuroendocrine cells and adrenal cells; pancreatic endrocrine cells, endocrine precursor cells, such as stem cells and duct cells; hepatocytes, and the like. Preferably, the cells enriched are differentiated endocrine cells or endocrine precursor cells, with insulin-secreting neonatal islet cells most preferred.

The source of tissue can be any type so long as it contains the selected or desired cells for enrichment, and/or contains precursor cells capable of becoming the desired cells during the culture process. For example, tissue can include all or part of the liver, pancreas, thyroid gland, reproductive glands, myocardial tissue, renal tissue, blood, and the like, or a preselected group of cells from within these organs. Most preferred as a tissue source is the pancreas, whereby it is isolated from a pig or human. Further, the source of the pancreatic tissue can be pre-adolescent, fetal or neonatal, with neonatal as the most preferred source.

The enriched population of preferred cells is substantially enriched as described herein. The term "substantially enriched" as used herein means a population of selected cells wherein the majority of or at least about 90% of the cells are the selected cell type. For example, enriched aggregates of neonatal islet cells contain about 5% or less fibroblasts or pancreatic exocrine cells and most preferably contain about 2-5% or less of such cells.

In the present method, the tissue is typically prepared by partial or full digestion, as necessary, using an agent or mixture of agents, such as proteases, collagenases, other enzymes, such as disphase, and the like to form a digest. Collagenase, such as Type V,

is a preferred agent and can be obtained commercially from various vendors, such as Sigma, Serva and Boehringer Mannheim. Other collagenases include liberase, Type P, and Type XI, which also can be purchased commercially. Alternatively, depending on the tissue source used, it may be desirable to use physical methods of preparing the enriched population of cells. For example, when blood is the tissue source, it may be desirable to use affinity chromatography.

During the culture step of the process, a serum-free basal media supplemented with various factors or agents is utilized. The agents include, but are not limited to, a serum substitute, an agent to stimulate DNA synthesis, and an agent to promote cell survival of the desired cell type. The media must be serum free; serum is omitted from the media due to its ability to promote the survival of contaminating or undesired cells, such as fibroblasts, pancreatic exocrine cells, and the like. Therefore, the serum substitute must not promote, enhance, or proliferate a contaminating cell type. Typically and most preferred, albumin is utilized as the serum substitute, with bovine serum albumin being the most common.

An agent or agents capable of stimulating DNA synthesis in the selected cell type or precursor is added to the media as a supplement. For example, isobutylmethylxanthene (IBMX) can be used for its capacity to stimulate DNA synthesis in islet cells and its potential to enhance the survival of cultured islets. Another DNA-synthesis stimulating agent is nicotinamide, wherein it has the ability to stimulate islet cell DNA replication and to positively affect the metabolic function of fetal or neonatal islet cells. Elevated glucose concentrations, *i.e.*, greater than or equal to 10mmol/liter, also stimulate DNA synthesis.

Also included as a supplement in the media is an agent capable of promoting cell survival of the cell desired for enrichment or capable of protecting the desired cell from destruction during the culture process. For example, glucose and/or IBMX can be used

as the cell survival promoting agent due to its cytoprotective effect of islet cells during culture and its ability to enhance islet cell replication.

The preferred supplements are present at about the following concentrations in the media: albumin from about 0.1% to about 1.0% weight/volume; IMBX from about 5 to about 100 µmol/liter; nicotinamide from about 0.5 to about 20 mmol/liter, and glucose from about 6 to about 30 mmol/liter. The digest is typically cultured at a temperature from about 20° to about 39°C for about 7 to about 20 days in humidified air. Most preferred, the digest is cultured for about 9 days at a temperature of about 37°C.

The serum-free tissue culture or basal media is a commonly used liquid tissue culture media that is free of serum. The media of the invention utilizes these media in combination with selected supplements or components to create a novel media to culture, enrich and allow the proliferation of, for example, the islet cells *in vitro*. Basal media useful in the culture method of the invention is any serum-free tissue culture media known in the art, including Media 199 (Gibco), CMRL 1066 (Gibco) media, Ham's F10 (Gibco) tissue culture media, and the like. These media also contain various ingredients, for example, amino acids, vitamins, inorganic salts, buffering agents, and energy sources. Purified molecules, such as hormones, growth factors, transport proteins, trace elements, antibiotics, and substratum-modifying ingredients optionally can be included in the media.

In a preferred embodiment, the invention comprises a serum-free basal media supplemented with albumin, IBMX, nicotinamide, and glucose. The pancreatic digest from one or more neonatal pig pancreas is preferably cultured with Ham's F10 tissue culture media, supplemented with about 10 m mol/liter of glucose, about 50 µmol/liter of IBMX, about 0.5% weight/volume of bovine serum albumin (BSA), and about 10 mmol/liter of nicotinamide. The media can optionally be supplemented with amino acids, such as L-glutamine, and antibiotics, such as penicillin and streptomycin. The

amount of L-glutamine is about 2 mmol/liter, whereas the amount of penicillin and of streptomycin is about 100 U/ml and 100 µg/ml respectively.

Once the substantially enriched population of cells is produced by the culture, it can be utilized as described below or the cells can be further isolated, purified, or matured.

The present invention provides methods for culturing a source of islet cells for use in transplantation. On average 250,000 islets are recovered from an 80-gram adult human pancreas, whereas, the instant invention allows the isolation of about 50,000 islets from a 2-3 gram neonatal pig pancreas. The resulting neonatal porcine islets can be transplanted into diabetic subjects; thereby improving their long-term prognosis.

In a preferred embodiment, a large-scale method of isolating neonatal islet cell (NIC) aggregates from a neonatal pancreas is provided. The method comprises digesting the pancreas with collagenase; and culturing the digest with a serum-free tissue culture or basal media supplemented with glucose, IBMX, albumin, and nicotinamide at about 37°C in humidified air for about nine days; resulting in neonatal islet cell aggregates. The source of the pancreas can be from any animal, including a human, but porcine pancreases are preferred.

The islet cells as described herein are useful for gene therapy methods as well. An exogenous or foreign gene of choice can be tranferred to the islet cells prior to transplantation. An "exogenous" or "foreign" gene refers to genetic material from outside the islet cell which is introduced into the cell. The term also includes a gene that has been modified from the native or natural form of a gene found in the cell to be transfected. For example, various cytokine genes or the like can be transfected into the cells by methods of transfection as calcium phosphate co-precipitation, conventional mechanical procedures such as microinjection, biolistics, insertion of a plasmid encased in liposomes, or by use of viral vectors. For example, one method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently

infect or transform the neuroblast (*Eukaryotic Viral Vectors*, Cold Spring Harbor Laboratory, Gluzman ed., 1982).

Various viral vectors which can be utilized for transfer of genes to the islet cells as taught herein include adenovirus, herpes virus, vaccinia, and preferably, an RNA virus such as a retrovirus. Retroviruses are useful particularly in the case of dividing cells. Therefore, the method of the invention, which provides a means for producing dividing and/or differentiated neonatal islet cells, provides cells that are susceptible to retrovirus infection. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). For human cells, preferably gibbon ape leukemia virus (GaLV) is utilized. A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated.

Since recombinant retroviruses are defective, they require assistance in order to produce infectious vector particles. This assistance can be provided, for example, by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus (*gag*, *env*, and *pol* genes) under the control of regulatory sequences within the long terminal repeat (LTR). These plasmids are missing a nucleotide sequence which enables the packaging mechanism to recognize an RNA transcript for encapsidation. Helper cell lines which have deletions of the packaging signal include, but are not limited to Ψ2, PA317, PA12, CRIP and CRE, for example. These cell lines produce empty virions, since no genome is packaged. If a retroviral vector is introduced into such cells in which the packaging signal is intact, but the structural genes are replaced by other genes of interest, the vector can be packaged and vector virion produced. The vector virions produced by this method can then be used to infect a tissue cell line, such as NIH 3T3 cells, to produce large quantities of chimeric retroviral virions.

Alternatively, NIH 3T3 or other tissue culture cells can be directly transfected with plasmids encoding the retroviral structural genes *gag*, *pol* and *env*, by conventional calcium phosphate or lipofection transfection. These cells are then transfected with the vector plasmid containing the genes of interest. The resulting cells release the retroviral vector into the culture medium.

The invention also envisions production of transgenic animals, *e.g.*, pigs, which would produce an unlimited supply of islet cells for transplantation. Cells removed from such pigs are cultured and isolated prior to transplantation (allotransplants) by the methods described herein. (See US Patent No. 4,736,866; EP 247,494)

The invention also describes a method of treating a subject having diabetes by administering to the subject a therapeutically-effective amount of islet cells, such as transplanting the *in vitro* insulin secreting neonatal islets described herein. "Therapeutically-effective" as used herein, refers to that amount of islet cells that is of sufficient quantity to alleviate a symptom of the disease or to ameliorate the diabetic disorder. "Ameliorate" refers to lessening or lowering the disease's or disorder's detrimental effect in the patient receiving the therapy. In the case of diabetics, the treatment can lower or eliminate the dependency on exogenous insulin.

The diabetic subjects or patients to be treated include animals, such as sheep, pigs, cats, rodents, cattle, non-human primates, and most preferably dogs and humans. The culture methods of the invention provide a source of neonatal islet cells from humans or other species, such as bovine, sheep, pigs, or non-human primates, with pigs and humans as preferred sources.

The invention also describes methods for transplantation, typically of xenografts, but also of allografts. Such methods include culturing neonatal islet cells to enrich islet endocrine cells (differentiated and/or undifferentiated), and then transplanting the resulting cells

into a suitable recipient. Glucose tolerance testing, as described herein, can be used to monitor the effectiveness of the islet transplant.

Any of the transplantation or implantation procedures known in the art can be utilized. For example, the selected cells or cells of interest can be surgically implanted into the recipient or subject. Further, the cells can be administered in an encapsulated form or non-encapsulated form.

Transplantation or implantation is typically by simple injection through a hypodermic needle having a bore diameter sufficient to permit passage of a suspension of cells therethrough without damaging the cells or tissue coating. For implantation, the typically encapsulated or coated cells are formulated as pharmaceutical compositions together with a pharmaceutically-acceptable carrier. Such compositions contain a sufficient number of coated transplant cells which can be injected into, or administered through a laparoscope to, an animal, usually into the peritoneal cavity if islet cells are utilized. However, other transplantation sites can be selected depending upon the cells and desired biological effect; these sites include the liver, spleen, kidney capsule, omental pouch, and the like.

Typically, the number of transplanted islets is from about 5 to about 10 thousand per kilogram of body weight. For example, in mice approximately 1,000-3,000 islets are transplanted. The number of other cells, tissues, and the like will be calculated depending on their function.

While not required, it may be desirable to administer an immunosuppressive agent to a recipient of the islet cells, prior to, simultaneous with, and/or after transplantation. An agent such as Cyclosporine A (CsA) is preferable, however other immune suppressive agents can be used, such as rapamycin, desoxyspergualine, and like. These agents are administered to cause an immunosuppressive effect in the subject, such that the transplanted islet cells are not rejected by that subject's immune system. Typically, the

immunosuppressive agent is administered continuously throughout the transplant treatment typically over a period of days or weeks; for example, CsA treatment ranges from about 2 to about 20 days at a dosage range of about 5 to 40 mg per kilogram of body weight per day. The agent can be administered by a variety of means, including parenteral, subcutaneous, intrapulmonary, oral, intranasal administration and the like. Preferably, dosing is given by oral administration.

Alternatively, the enriched cells also can be encapsulated prior to transplantation. Although the cells are typically microencapsulated, they can be encased in various types of hollow fibers or in a block of encapsulating material. Encapsulation provides an effective protective barrier to isolate the transplanted cells or tissues from the host or recipient's immune system. Typical pharmaceutical encapsulation compositions include, *e.g.*, liposomes, gelatin, polyvinyl alcohol, ethylcellulose, cellulose acetatephthalate and styrene maleic anhydride. *See* Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton PA (1990).

A variety of microencapsulation methods and compositions are known in the art. A number of microencapsulation methods for use in transplant therapy have focused on the use of alginate polymers or agarose to supply the encapsulation compositions. Alginates are linear polymers of mannuronic and guluronic acid residues which are arranged in blocks of several adjacent guluronic acid residues forming guluronate blocks and block of adjacent mannuronic acid residues forming mannuronate blocks, interspersed with mixed, or heterogenous blocks of alternating guluronic and mannuronic acid residues. Generally, monovalent cation alginate salts are soluble, *e.g.*, Na-alginate.

Divalent cations, such as Ca^{++}, Ba^{++} or Sf^{+}, tend to interact with guluronate, and the cooperative binding of these cations within the guluronate blocks provides the primary intramolecular crosslinking responsible for formation of stable ion-paired alginate gels. Alginate encapsulation methods generally take advantage of the gelling of alginate in the presence of these divalent cation solutions. In particular, these methods involve the

suspension of the material to be encapsulated, in a solution of monovalent cation alginate salt, *e.g.*, sodium. Droplets of the solution are then generated in air and collected in a solution of divalent cations, *e.g.*, $CaCl_2$. The divalent cations interact with the alginate at the phase transition between the droplet and the divalent cation solution resulting in the formation of a stable alginate gel matrix being formed. Generation of alginate droplets has previously been carried out by a number of methods. For example, droplets have been generated by extrusion of alginate through a tube by gravitational flow, into a solution of divalent cations. Similarly, electrostatic droplet generators which rely on the generation of an electrostatic differential between the alginate solution and the divalent cation solution have been described. The electrostatic differential results in the alginate solution being drawn through a tube, into the solution of divalent cations. For a general discussion of droplet generation in encapsulation processes, see, *e.g.*, M.F.A. Goosen, Fundamentals of Animal Cell Encapsulation and Immobilization, Ch. 6, pp. 114-142 (CRC Press, 1993).

Further, methods have been described wherein droplets are generated from a stream of the alginate solution using a laminar air flow extrusion device. Specifically, this device comprises a capillary tube within an outer sleeve. Air is driven through the outer sleeve and the polymer solution is flow-regulated through the inner tube. The air flow from the outer sleeve breaks up the fluid flowing from the capillary tube into small droplets. See U.S. Patent No. 5,286,495.

Microencapsulation also has been applied in the treatment of diseases by transplant therapy. While traditional medical treatments for functional deficiencies of secretory and other biological organs have focused on replacing identified normal products of the deficient organ with natural or synthetic pharmaceutical agents, transplant therapy focuses on replacement of that function with cell or organ transplants. For example, the treatment of insulin-dependent diabetes mellitus, where the pancreatic islets of Langerhans are nonfunctional, can be carried out by replacing the normal secretion of insulin by the islets in the pancreas. Insulin may be supplied either by daily administra-

tion of synthetic or substitute animal insulin, or by transplantation of functional human or animal islets.

Attempts to transplant organ tissues into genetically dissimilar hosts without immunosuppression are generally defeated by the immune system of the host. Accordingly, attempts have been made to provide effective protective barrier coatings, *e.g.*, by microencapsulation, to isolate the transplant tissues from the host immune system. Successful cell or tissue transplants generally require a coating that will prevent their destruction by a host's immune system, prevent fibrosis, and will be permeable to and allow a free diffusion of the nutrients to the coated transplant and removal of the secretory and waste products from the coated transplant.

Viable tissue and cells have been successfully immobilized in alginate capsules coated with polylysine. See *J. Pharm. Sci.* 70:351-354 (1981). The use of these coated capsules in pancreatic islet transplantation to correct the diabetic state of diabetic animals has also been described in *Science* 210:908-909 (1981).

The prolonged reversal of the diabetic state of mice with xenografts of microencapsulated rat islets, using alginate-polylysine capsules has been reported. See *Diabetes* 40:1511-1516 (1993). The development of transplants encapsulated in calcium alginate capsules reacted with polylysine is also described, for example, in U.S. Patent Nos. 4,673,566, 4,689,293, 4,789,550, 4,806,355, and 4,789,550.

U.S. Patent 4,744,933 describes encapsulating solutions containing biologically active materials in a membrane of inter-reacted alginate and polyamino acid.

U.S. Patent 4,696,286 reports a method for coating transplants suitable for transplantation into genetically dissimilar individuals. The method involves coating the transplant with a surface conforming bonding bridge of a multi-functional material that binds chemically to a surface component of the transplant, which is enveloped in a semiperme-

able, biologically compatible layer of a polymer that binds chemically to the bonding bridge layer.

A method for introducing a second alginate gel coating to cells already coated with polylysine alginate is described in U.S. Patent 5,227,298. Both the first and second coating of this method require stabilization by polylysine.

Encapsulation methods applied to make these materials have comprised a procedure for forming droplets of the encapsulating medium and the biological material and a procedure for solidifying the encapsulating medium. Agarose encapsulated materials have been formed by chilling an emulsion of agarose droplets containing biological materials as shown by Nilsson, *et al.*, *Nature* 302:629-630 (1983) and Nilsson, *et al.*, *Eur. J. Appl. Microbiol. Biotechnol.* 17:319-326 (1983). Injection of droplets of polymer containing biological materials into a body of coolant such as concurrently liquid stream has been reported by Gin, *et al.*, *J. Microencapsulation* 4:329-242 (1987).

It is further envisioned that an undifferentiated cell that is microencapsulated or encapsulated with a stabilizing matrix, *i.e.*, block or hollow fibers, will mature at a rate faster than that of an unencapsulated cell. "Maturation" as used herein, means the ability of a cell to differentiate or to achieve a specific biological function or metabolic activity. Therefore, an encapsulated cell will mature at an increased rate over a cell not so encapsulated. Typically, the maturation rate will be at least about a 2-fold increase. Similarly, when an increase in proliferation rate is desirable, the rate will be at least about a 2-fold increase. Not being limited to a particular theory, it is believed that microencapsulation prevents cell aggregation thereby eliminating central necrosis of the aggregates, and allows the enclosed cells to grow and mature during culture. Another aspect of this invention is that encapsulation of a cell also increases the cell's rate of growth or proliferation in culture. For example, enclosing the neonatal islet cells within a microcapsule or stabilizing matrix allows them to mature, grow, and differentiate into insulin-secreting cells during the *in vitro* culture stage as well as after implantation.

Further, once the cell is encapsulated it can be cultured *in vitro* in either a serum-containing media or serum-free media.

In another aspect of the invention, more than one cell type can be co-encapsulated in the capsule. This includes both naturally isolated or genetically manipulated cells as described herein. Cell types may include distinct stages of a single lineage of cells (*e.g.*, both precursor and differentiated endocrine cells) or distinct cell lineage (*e.g.*, endocrine and blood)

Therefore, the culture of any cell type, including neonatal, fetal, and/or pre-adult cells, in such a protective growth-enhancing environment results in a increased rate of maturation or proliferation. Cell types include endocrine cells, such as neuroendocrine and adrenal cells; pancreatic endrocine cells, endocrine precursor cells, such as stem cells and duct cells; hepatocytes, cholinergic neurons, hematopoietic cells, hippocampal cells and the like. Other cell types that can be cultured within the microcapsule or matrix include, but are not limited to, any neonatal, fetal, pre-adolescent, adult, or other cell type capable of maturation and/or proliferation.

The following examples are intended to illustrate, but not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be used.

Examples

The aims of the present investigation were to develop a standardized method for the large scale isolation of porcine neonatal islet cell (NIC) aggregates, to define the cellular composition of these aggregates, and to assess their growth potential and viability both *in vitro* and *in vivo*. The present invention shows the feasibility of using the neonatal porcine pancreas as a source of insulin-producing tissue for xenotransplantation into human Type I diabetics.

Based upon existing methods of isolating fetal porcine islet tissue, a simple, reliable procedure was developed for the preparation of porcine neonatal islet cell aggregates with a reproducible and defined cellular composition. Following 9 days of *in vitro* culture, tissue from one neonatal pig pancreas yielded approximately 50,000 islet cell aggregates, consisting of primarily epithelial cells (57%) and pancreatic endocrine cells (35%). During the culture period, the total cell mass decreased initially, but subsequently increased 1.5-fold between days 3 and 9. Transplantation of grafts consisting of 3×10^5 cells (1000 aggregates) under the kidney capsule of alloxan-diabetic nude mice corrected hyperglycemia in 75% (10/13) of the animals, whereas, 100% (20/20) of recipients implanted with 6×10^5 cells (2000 aggregates) achieved euglycemia within 8 weeks post-transplantation. Nephrectomy of the graft bearing kidney at 14 weeks posttransplantation resulted in hyperglycemia in all recipients, and examination of the grafts revealed the presence of numerous well-granulated insulin- and glucagon-containing cells. The cellular insulin content of these grafts was 20 to 30-fold higher than at the time of transplantation. Further, aggregates cultured in alginate microcapsules and then transplanted into diabetic recipients corrected the diabetes within 1-7 days post-transplantation. These results indicate that the neonatal porcine pancreas can be used as a source of large numbers of viable islet cells, which have the potential for growth both *in vitro* and *in vivo*, and exhibit the metabolic capacity to correct diabetes.

EXAMPLE 1

MATERIALS AND METHODS

Media and Products.

The isolation of porcine NIC aggregates was carried out in Hank's balanced salt solution (HBSS; Gibco, Burlington, Ont.) supplemented with 0.25% bovine serum albumin (BSA; fraction V, Sigma, St. Louis, MO), 10 mmol/l HEPES (ICN Biomedicals, Inc., Costa Mesa, CA), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Ham's F10 tissue culture medium was purchased from Gibco, isobutylmethylxanthine (IBMX) from ICN Biomedicals, nicotinamide from BDH Biochemical (Poole, England) and theophylline from Aldrich (Milwaukee, WIS). Insulin release experiments were carried out in RPMI tissue culture medium (Gibco). Collagenase (Type V) was obtained from Sigma, and crystalline trypsin, bovine pancreatic DNase, Proteinase K, and RNase A from Boehringer Mannheim (Laval, Quebec). Picogreen (P-7581), a fluorescent nucleic acid stain for quantification of double-stranded DNA was purchased from Molecular Probes (Eugene, OR).

Animals.

Donor pancreases were obtained from 1-3 day old Landrace-Yorkshire neonatal pigs (1.5-2.0 kg body weight) of either sex. Piglets were anesthetized with Halothane and subjected to laparotomy and complete exsanginuation. The pancreas was then carefully dissected from surrounding tissue and placed in cooled (4°C) HBSS (supplemented as above). Warm and cold ischemia was kept to <10 and <5 min., respectively. Eight glands were initially used to standardize the procurement and isolation procedure. Data were then obtained from 10 consecutive independent experiments, with islet cells prepared from 3 neonatal pig pancreases for each experiment.

Male, inbred, athymic nude Balb/c mice (age 6-8 weeks) were used as recipients of the NIC aggregates (Jackson Laboratories, Bar Harbour, Ma). Mice were rendered diabetic by intravenous injection of 90 mg/kg body wt alloxan (Sigma; freshly dissolved in 1 mmol/l hydrochloric acid) 4 to 5 days before transplantation. Normoglycemic, a-

ge-matched mice served as normal controls. All recipients entering this study exhibited blood glucose levels above 20 mmol/l. Blood samples were obtained from the tail vein for glucose assay (Medisense glucose meter, Medisense Canada, Mississauga, Ontario). Animals were maintained under Virus Antibody Free conditions in climatized rooms with free access to sterilized tap water and pelleted food.

Preparation and Culture of porcine NIC aggregates.

Each of the glands were cut into fragments of approximately 1-2 mm^3, then transferred to sterile tubes containing HBSS (supplemented as above) with 2.5 mg/ml collagenase, and gently agitated for 16-18 min. in a shaking water bath at 37°C. The digest was filtered through a nylon screen (500 um), washed four times in HBSS then placed into bacteriological petri dishes containing HAM's F10 tissue culture medium (10 mmol/l glucose, 50 umol/l IBMX, 0.5% BSA, 2 mmol/l L-glutamine, 10 mmol/l nicotinamide, 100 U/ml penicillin and 100 ug/ml streptomycin). Culture dishes were maintained at 37°C (5% CO2, 95% air) in humidified air for 9 days, with the medium and dishes changed the first day after isolation and the medium every second day thereafter.

For some experiments, following 8 days of culture, approximately 50% of the NIC aggregates from each preparation were microencapsulated with highly purified alginate (Metabolex, Inc.) producing uniform capsules of 250 to 350 µm in diameter. Encapsulated and non-encapsulated aggregates were then further cultured for 8 days (HAM's F10; 37°C) in the presence and absence of 5% (v/v) heat-activated autologous neonatal pig serum. Controls consisted of non-encapsulated aggregates cultured for 16 days in serum free HAM's F10.

Characterization of islet cell preparations.

Following the isolation procedure, and after 3 and 9 days of tissue culture, recovery and purity of the NIC aggregates was determined on the basis of cellular hormone, DNA, and amylase content. All measurements were assessed from duplicate aliquots of the islet cell suspensions. Hormone content was measured after extraction in 2 mmol/l acetic acid containing 0.25% BSA. Samples were sonicated in acetic acid, centrifuged (800 x g, 15

min.), then supernatants were collected and stored at -20°C until assayed for insulin content by ELISA (Boehringer Mannheim) and for glucagon content by means of radioimmunoassay (Diagnostic Products Corp.; Los Angeles, CA). Amylase content was determined in supernatant fractions collected from cell suspensions which were sonicated in supplemented HBSS, stored at -20°C, then measured by an enzymatic amylase assay (Beckman, Carlsbad, CA). For DNA content, aliquots were washed in citrate buffer (150 mmol/l NaCl, 15 mmol/l citrate, 3 mmol/l EDTA, pH 7.4) and stored as cell pellets at -20°C. Prior to being assayed, cell pellets were placed in 450ul of lysis buffer (10 mmol/l Tris, 1 mmol/l EDTA, 0.5% Triton X-100, 4°C, pH 7.5), sonicated, supplemented with 25 ul of Proteinase K solution (8 mg/ml), vortexed, and incubated at 65 and 70°C for 45 and 10 min., respectively. Lysates were supplemented with 25 ul of RNase A solution (10 mg/ml), vortexed, and incubated for 1 h at 37°C. Aliquots of 25 and 50 ul were assayed in duplicate by diluting them in 1 ml of DNA buffer (10 mmol/l Tris, 1 mmol/l EDTA, pH 7.5) and measuring fluorescence at 490 exc. / 515 em. nm following the addition of 1 ml of Pico Green reagent (1/200 dilution with DNA buffer). Samples were run in parallel with and diluted in proportion to a seven point (0 - 400 ng/ml) standard curve which was generated using calf thymus DNA. In order to determine DNA content per islet cell, NIC aggregates were dissociated into single cell suspensions by gentle agitation in calcium-free medium containing trypsin (15 ug/ml) and DNase (4 ug/ml, ref. 27-29). A Burker chamber was used for cell counts (27,28), and samples ranging from 2 - 5 x 10^4 cells were assayed for DNA content.

The cellular composition of each fraction was determined by electron microscopy and immunohistochemistry using methods similar to that previously described by Pipeleers et al (30-32). Aggregates were fixed in 2.5% (v/v) glutaraldehyde (Millonig's buffer, pH 7.2), post-fixed in 1.5% (w/v) OsO4, washed in distilled water, then dehydrated successively in 50, 70, 80, 90 and 100% ethanol, prior to embedding in araldite. For electron microscopy, sections were stained with lead citrate and uranyl acetate then subsequently examined in a Hitachi H 7000 transmission electron microscope. In each sample, minimally 100 cells were examined and characterized as exocrine (presence of

zymogen granules >500 nm in diameter), endocrine (presence of smaller granules typical for a, b, d, or pancreatic polypeptide cells), nongranulated (absence of secretory vesicles), or as damaged (ruptured plasma membranes and/or swollen organelles) (30,31). For immunohistochemistry, the avidin-biotin complex (ABC) method was used with peroxidase and diaminobenzadine as the chromagen. Sections (1 um) were affixed to glass slides by heat, the plastic resin removed with sodium methoxide and counter stained with Harris's hemotoxylin for 2 min., then subsequently stained separately for the presence of insulin- and glucagon-containing cells. In each experiment, a minimum of 15 aggregates randomly selected from 3 to 4 different sections were examined. Primary antibodies (Dako; Carpinteria, CA) included, guinea pig anti-porcine insulin (1:1000) and rabbit anti-glucagon (1:100); biotinylated secondary antibodies and the ABC-enzyme complexes were purchased from Vector Laboratories (Burlingame, CA). Primary antibodies were incubated for 30 min. (room temperature), while secondary antibodies were applied for 20 min.

For assessment of *in vitro* viability, the NICs secretory response to glucose was determined following 9 days of tissue culture by using a static incubation assay (27). The cultured fractions were recovered from the Petri dishes, washed and aliquots of 50 to 100 aggregates were incubated for 120 min. in 1.5 ml of RPMI medium supplemented with 2 mmol/l L-glutamine, 0.5% BSA and either 2.8 mmol/l glucose, 20 mmol/l glucose or 20 mmol/l glucose plus 10 mmol/l theophylline. Tissue and medium were then separated by centrifugation and assayed for their respective insulin contents. The insulin content of the medium was expressed as a percentage of the total content (*i.e.* tissue plus medium). Stimulation indices were calculated by dividing the amount of insulin release at 20 mmol/l glucose (+/- theophylline) by that released at 2.8 mmol/l glucose. In four independent experiments a portion of the freshly isolated NIC preparation was cultured for 9 days in the supplemented HAM's F10 medium, but without the addition of 10 mmol/l nicotinamide, in order to assess whether nicotinamide influenced the insulin secretory capacity of porcine neonatal β cells.

Transplantation and metabolic follow-up.

Following 9 days of culture, NIC aggregates were transplanted under the left kidney capsule of Halothane-anesthetized nude mice. Prior to implantation, the cellular composition of the graft was characterized as outlined above, and in order to standardized the mass of islet cells transplanted in each experiment, representative aliquots of each preparation were counted, sized, and the final quantity of aggregates was converted to the number equivalent to a diameter of 150 um (33). Aliquots consisting of 1000 or 2000 aggregate equivalents were aspirated into polyethylene tubing (PE-50), pelleted by centrifugation, and gently placed under the kidney capsule with the aid of a micromanipulator syringe. Once the tubing was removed, the capsulotomy was cauterized with a disposable high-temperature cautery pen (Aaron Medical Industries, St. Petersburg, FL.).

Transplanted mice and normal controls were monitored for blood glucose levels once a week between 8:00 and 11:00 a.m. When the blood glucose level was ≤8.4 mmol/l, the graft was deemed a success. At posttransplantation week 12, an oral (OGTT) and then an intraperitoneal (IPGTT) glucose tolerance test 48 h later, was performed on NIC recipients with normalized basal glycemia and in normal controls. After a 2-h fast, D-glucose (3 mg/gm body weight) was administered as a 50% solution intragastricly or injected intraperitonealy into non-anesthetized mice. Blood samples were obtained from the tail vein at 0,15, 30, 60, and 120 min.

In another set of experiments, following 16 days of culture, the alginate microcapsule was removed from some of the aggregates by incubating the encapsulated aggregates in calcium-free HBSS containing 0.5% BSA and 1 mmol/l ethylene glycol-bis-(β-amino ethyl ether) $N^{l}N^{l}$-tetra acetic acid for 30 min. Non-encapsulated aggregates were also treated in the same manner. Prior to implantation, in order to standardized the mass of islet cells transplanted in each experiment, representative aliquots of each preparation were counted, sized, and the final quantity of aggregates was converted to the number equivalent to a diameter of 150 μm. Aliquots of 2000 aggregate equivalents, both encapsulated and non-encapsulated, were aspirated into polyethylene tubing (PE-50),

pelleted by centrifugation, and gently placed under the kidney capsule of Halothane-anesthetized nude mice with the aid of a micromanipulator syringe. Once the tubing was removed, the capsulotomy was cauterized with a disposable high-temperature cautery pen (Aaron Medical Industries, St. Petersburg, FL.). Transplanted mice were monitored for blood glucose levels once a week between 8:00 and 11:00 am. When the blood glucose level was ≤8.4 mmol/l, the graft was deemed a success.

Characterization of harvested NIC grafts.

At 14 weeks posttransplantation, NIC recipients underwent a nephrectomy of the graft-bearing kidney for morphological analysis or to determine insulin and glucagon contents of the harvested grafts. The grafts in four recipients receiving 2000 aggregates were however, not removed at this time, and these animals were monitored for an additional 7 months. Nephrectomized animals were subsequently monitored to confirm a return of hyperglycemia. The graft-bearing kidneys were immersed in Bouin's solution overnight and embedded in paraffin. Sections, 5 um thick, were then stained for the presence of insulin and glucagon containing cells, as described above. Pieces of native neonatal pig pancreas were also processed and analyzed according to this procedure. In two recipients, the graft and adjacent kidney tissue was fixed in glutaraldehyde then processed for electron microscopy. For hormone extraction, organs were homogenized and then sonicated at 4°C in 10 ml of 2 mmol/l acetic acid (0.25% BSA). Following 2-h at 4°C, tissue homogenates were re-sonicated, centrifuged (8000 x g, 20 min.), then supernatants were collected and the pellets further extracted by sonication in an additional 8 ml of acetic acid. The second supernatant was collected after centrifugation, combined with the first supernatant, total volume was measured, and samples were assayed for insulin and glucagon content. The same procedure was also used to extract hormones from pancreases obtained from NIC recipients, normal control mice, and 1-3 day old neonatal pigs.

Statistical Analysis.

Data are expressed as means ± SE of n independent observations. Statistical significance of differences was calculated with a two-tailed unpaired Student's t test or a one-way analysis of variance in case of multiple comparisons.

EXAMPLE 2

PORCINE NIC PREPARATION

Following collagenase digestion, a mean of 134 ug of insulin was recovered per neonatal pancreas (Table I). The digest contained approximately 65% of the total insulin content present in nondigested 1-3 day old neonatal pig pancreases, which were found to contain 205±7 ug insulin (range = 191-242 ug). Tissue culture resulted in further losses of cellular insulin mass, so that after 3 and 9 days of culture, 72 and 64% of the amount initially present in the digest was recovered, respectively (Table I). On the other hand, culture caused a marked reduction in the amount of recoverable DNA, as less than 20% of that found in the digest was present after 3 days of culture and only 10% following nine days ($p<0.0001$, Table I). Similarly, cultured preparations exhibited amylase values lower than 1% of the digests (Table I). The insulin content per microgram of DNA or per amylase content (U) of the porcine NICs significantly increased during culture (Table I). On the basis of the insulin per DNA values, 9 day cultured NIC preparations were more than 6-fold ($p<0.0001$) enriched in endocrine tissue compared to the freshly isolated material (Table I). When expressed as a ratio of insulin per amylase, enrichment in endocrine tissue was more than 900-fold ($p<0.0001$) after the 9 day culture period (Table I).

Electron micrographs of the freshly isolated preparations indicated that >90% of the cells were nonendocrine, of which the majority were exocrine (74%) and only 7% were shown to contain secretory vesicles characteristic of endocrine cells (Table II). Immunohistochemical analysis of the digest demonstrated the presence of 5% insulin-positive and 2% glucagon-positive cells (Table II). During the first 2 days of culture, many degenerating cells were observed cleaving away from the edges of a centrally located islet cell

aggregate. Microscopically, NIC aggregates developed into spherical structures by the third day of culture and began to exhibit a translucent appearance similar to adult pancreatic islets. Tissue culture markedly reduced the percentage of exocrine cells, so that at 9 days post-isolation, <5% of the cells were identified as exocrine (Table II). These morphological findings are therefore consistent with the observed reduction in amylase content following culture. The nine day cultured preparations consisted of 35% structurally intact endocrine cells containing well-developed endoplasmic reticulum (Fig. 1). This percentage of endocrine cells was significantly higher than at the start of culture ($p<0.001$; Table II). In general, 9 day cultured aggregates contained numerous non-granulated epithelial cells (57%) , as well as many duct-like structures were found in the aggregates (Fig. 1). A low degree of cellular damage was also observed in the electron micrographs (Table II). Immunohistological examination confirmed that 9 day cultured preparations consisted mainly of epithelial cells with the presence of 24% insulin-containing and 8% glucagon-containing cells scattered randomly throughout the aggregate (Fig. 2c and 2d). This random distribution of endocrine cells was also similar to that observed in the native neonatal porcine pancreas (Fig. 2a and 2b).

The quantity of aggregates recovered in each experiment was estimated using the method previously described for determining human islet equivalents (33). The mean yield obtained from one neonatal porcine pancreas following collagenase digestion and 9 days culture was 48,526±3125 aggregates (range = 28,210-90,966). Approximately 50% of the aggregates measured between 50 and 99 um, 40% were 100-149 um, and the remainder were either <50 um or ranged from 150-250 um.

EXAMPLE 3

INSULIN SECRETORY RESPONSIVENESS

The secretory activity of NIC aggregates cultured for 9 days in the presence or absence of 10 mmol/l nicotinamide was tested by comparing the percentages of cellular insulin that was released at low glucose (2.8 mmol/l), high glucose (20 mmol/l) and high glucose plus theophylline (10 mmol/l). No statistically significant differences were noticed in

the amounts of insulin secreted at the low glucose concentration (Table III). Incubation in 20 mmol/l glucose significantly ($p<0.001$) increased the secretory rate of both nicotinamide treated and nontreated NICs; this effect was further potentiated when the NICs were exposed to high glucose plus theophylline (Table III). However, the amount of insulin released and the calculated stimulation indices after incubation with either 20 mmol/l glucose or 20 mmol/l glucose plus 10 mmol/l theophylline were significantly higher when the NICs were previously cultured with nicotinamide. Nicotinamide treated aggregates exhibited stimulation indices >5-fold when comparing insulin release at high glucose versus that at low glucose. When exposed to 20 mmol/l glucose in combination with 10 mmol/l theophylline, stimulation indices were >39-fold (Table III). Analysis of the total cellular insulin and DNA content recovered per NIC aggregate, and the percentage of insulin-positive cells, revealed no significant differences between nicotinamide treated and untreated preparations.

EXAMPLE 4

COMPOSITION OF NIC GRAFTS

Prior to transplantation, the composition of 9-day cultured NIC grafts was determined by electron microscopy, immunohistochemistry, hormone content, and DNA assay (Tables II and IV). As previously described, the NIC grafts were composed of 57% non-granulated and 35% endocrine cells; the remaining cell types were identified as either exocrine (3%) or damaged cells (5%; Table II). Immunohistochemical staining for insulin and glucagon was positive for 24 and 8% of the total cell population, respectively (Table II). On the basis of the implants DNA content (Table IV) and the observation that single porcine neonatal islet cells prepared from 9-day cultured aggregates were found to contain 7.1 pg DNA/cell (data not shown), it was calculated that grafts consisting of 1000 or 2000 aggregates contained 1.3 or 2.6 million cells, respectively. In view of the percentage of insulin-positive cells, the implants should therefore contain approximately 0.3 or 0.6 x 10^6 insulin-producing β cells. The measurement of DNA, insulin, and

glucagon content also confirmed that the mass of the 2000 aggregate grafts was correspondingly larger than the 1000 aggregate grafts.

EXAMPLE 5

TRANSPLANTATION OF NIC AGGREGATES INTO DIABETIC NUDE MICE

After alloxan administration, all NIC recipients exhibited blood glucose levels above 20 mmol/l. Diabetic controls (n=9) not receiving a graft were shown to survive for 11±4 days. All animals transplanted with 2000 NIC aggregates exhibited blood glucose values ±8.4 mmol/l within 8 weeks posttransplantation (Table V). This metabolic state was maintained over the 14 week follow-up period, and in 4 animals which were not sacrificed at 14 weeks, normoglycemia has been maintained for more than 11 months posttransplantation. On the other hand, 10 of the 13 animals implanted with 1000 aggregates achieved normoglycemia (blood glucose ±8.4 mmol/l) within the follow-up period (Table V). At 14 weeks posttransplantation, blood glucose values of those 1000 aggregate recipients not obtaining euglycemia were: 10.7, 12.2, and 14.4 mmol/l. Comparison with normal controls indicated that recipients of 2000 NIC aggregates exhibited significantly lower blood glucose levels at weeks 12 and 14 (Table V). These values did not, however, continue to decrease further, as those recipients (n=4) allowed to survive long term exhibited glucose levels of 5.2±0.6 at 11 months posttransplantation ($p<0.01$ vs. normal controls). No differences were measured between the mean glucose values of the 10 normoglycemic 1000-aggregate recipients and normal controls (Table V). In both transplanted groups, removal of the graft-bearing kidney for morphological examination or hormone extraction was followed by a rapid return to the diabetic state, indicating that the NIC grafts were responsible for the normoglycemic state.

Glucose tolerance tests were performed on normoglycemic mice 12 weeks post-transplantation, and when compared to normal control mice, recipients of 2-000-aggregates exhibited significantly lower glycemic values at all time points (Fig. 3). When the 1000-aggregate recipients were compared to normal controls, their blood

glucose levels were not statistically different throughout both tests. Comparison of the two transplant groups indicated that the 1000-aggregate recipients exhibited statistically higher values at 15 and 30 min. in the OGTT and at 15, 30, and 60 min. for the IPGTT (Fig. 3). In all groups, the glycemia at min. 120 after the bolus of glucose was not significantly different from the values at min. 0.

EXAMPLE 6

HORMONE CONTENT AND MORPHOLOGICAL CHARACTERIZATION OF NIC GRAFTS

Before implantation, NIC grafts contained on average 1.9 ug of insulin/1000 aggregates or 4.0 ug of insulin/2000 aggregates (Table IV). Fourteen weeks posttransplantation, considerably larger quantities of cellular insulin were recovered from the graft bearing kidneys (Table VI). There was however, no difference in the glucagon content between the harvested grafts and the grafts analyzed at the time of implantation (Table VI). The insulin content of grafts obtained from normoglycemic recipients implanted with 1000 aggregates contained 30-fold (63.7 ug) more insulin than what was initially transplanted (Table VI). Grafts retrieved from animals receiving 1000 aggregates and which did not achieve euglycemia, were shown to contain 12.2, 16.7, and 18.9 ug insulin. In animals transplanted with 2000 aggregates, grafts contained more than 20-fold (88 ug) more insulin than at the time of implantation (Table VI). The amount of insulin extracted from grafts obtained from recipients of 1000 and 2000 aggregates corresponds to, respectively, more than 74 and 141% of the pancreatic insulin content in aged-matched normal control mice (Table VI). Glucagon content, on the other hand, was similar to that found in pancreases of normal controls. The recipients pancreatic insulin content was less than 1% of that contained in normal control animals, whereas their glucagon content was similar to that in normal controls (Table VI).

Macroscopically, considerable growth of the NIC grafts was evident following 14 weeks posttransplantation. Immunohistological examination of the grafts revealed a highly vascularized tissue, consisting predominantly of well-granulated insulin- and glucagon-containing cells (Fig. 2 e,g and 2 f,h respectively). Epithelial cells were not frequently seen in the grafts. The β cells, which composed the major volume of the graft, were arranged in ductal-/tubular-like structures and the endocrine non-β cells were scattered randomly amongst the β cells. No marked differences in morphology were observed between the two transplant groups. In electron micrographs, donor endocrine

cells were shown to be structurally intact, highly granulated, and mitotic activity was detected within some of the grafts β-cells (Fig. 4).

EXAMPLE 7

MICROENCAPSULATION OF ISLET CELLS

Eight-day cultured aggregates were microencapsulated with purified alginate, then recultured for 8 days in the presence or absence of 5% (v/v) non-heat inactivated autologous serum, as described in EXAMPLE 1. Controls included nonencapsulated islets cultured in the same manner. In serum free medium, the number of cells and the amount of insulin recovered from nonencapsulated islets decreased by 38±3 and 69±4%, respectively. Moreover, when nonencapsulated islets were cultured with serum, extensive clumping and central necrosis occurred, causing significant loss (>90%) of islet cell mass and insulin content. When encapsulated islets were cultured in the absence of serum, the cell number decreased by 16±2%, where as the amount of recoverable insulin increased by 14±2%. Addition of serum to the culture medium of encapsulated preparations, increased the insulin content by 89±6%, but had no effect on cell number. Transplants were then carried out in diabetic nude mice, and recipients were monitored for the time required to achieve euglycemia (Table VIII). All mice (n=12) implanted intraperitoneally with 2000 encapsulated islets cultured with serum, became normoglycemic within 14 days posttransplantation (range 3-14 days). Similarly, mice transplanted under the kidney capsule (n=9) with grafts obtained by dissolving the alginate capsule after 8 days culture in serum, obtained euglycemia within 2 weeks. In contrast, animals transplanted with 2000 non-encapsulated (renal subcapsular space; n=8) or encapsulated (intraperitoneally; n=10) islets cultured in the absence of serum required minimally 4-8 weeks to normalize blood glucose levels.

EXAMPLE 8

CO-ENCAPSULATION OF ISLETS WITH SERTOLI CELLS

In order to create an ectopic site, two thousand allogenic rat islets (Lewis) were co-encapsulated with allogenic Sertoli cells (Lewis) and implanted intraperitoneally into streptozotocin-induced diabetic (glycemia >20 mM) Wistar-Furth recipients (Table IX). All animals receiving encapsulated islet grafts without Sertoli cells achieved euglycemia (glycemia <8.4mM) within 2 days, but all returned to a diabetic state by 8-14 days posttransplantation (mean survival time 10.4±0.6 days). When islets were co-encapsulated with Sertoli cells, all animals again normalized within 2 days, and remained euglycemia for a period ranging from 74-98 days posttransplantation (mean survival time 85.0±4.9 days). These results demonstrate, that creation of a clinically applicable ectopic site that co-localizes islets and Sertoli cells, leads to a long-term protection of an islet allograft without the need for systemic immunosuppression.

Table I. Preparation of porcine neonatal islet cell aggregates.

	Recovery per Pancreas			Purity	
Culture Period	Insulin (μg)	DNA (μg)	Amylase (U)	Insulin (μg)/DNA (μg)	Insulin (μg)/Amylase (U)
Freshly isolated	134 ± 12	4140 ± 464	60.03 ± 5.40	0.032 ± 0.002	2.2 ± 0.3
3 Days	96 ± 7*	753 ± 32‡	0.42 ± 0.01‡	0.129 ± 0.010‡	222.1 ± 16.0‡
9 Days	86 ± 8†	394 ± 24†	0.04 ± 0.001‡	0.215 ± 0.011†§	2121.1 ± 77.6†§

Values are means ± SE of 10 independent experiments. In each experiment, porcine NIC aggregates were prepared from 3 pancreases. Statistical significance of differences was calculated by one-way analysis of variance.

*$p<0.05$, †$p<0.01$, ‡$p<0.0001$ vs. freshly isolated.

§$p<0.0001$ vs. 3 Days.

Table II. Cellular composition of porcine neonatal islet cell aggregates during tissue culture.

		Cell composition (% of total)				Cell type (% positive)	
Culture Period	n	Endocrine	Nongranulated	Exocrine	Damaged	Insulin	Glucagon
Freshly isolated	7	7±2	11±1	74±2	8±1	5±1	2±1
3 Days	5	11±3	45±3‡	41±3‡	4±1*	8±1	3±1
9 Days	7	35±5†§	57±5‡¶	3±1‡¶	5±1	24±3‖"	8±1‡"

Values are means ± SE of n independent determinations. In each experiment, porcine NIC aggregates were prepared from 3 pancreases.

Cell composition was determined in electron micrographs and the percent glucagon and insulin containing cells was determined by immunohistochemistry, as described in Methods. Statistical significance of differences was calculated by one-way analysis of variance.

*$p<0.05$, †$p<0.001$, ‡$p<0.0001$ vs. freshly isolated.

§$p<0.01$, "$p<0.001$, ¶$p<0.0001$ vs. 3 Days.

Table III. Effect of nicotinamide on insulin secretory capacity of porcine neonatal islet cell aggregates following nine days of culture.

		Isolated islet fraction (insulin secretory activity (% content)			Stimulation Indices	
Condition	n	2.8 mmol/l glucose	20 mmol/l glucose	20 mmol/l glucose + 10 mmol/l theophylline	High:low	High + theophylline:low
Ham's F10						
Nicotinamide	10	0.9±0.1	4.8±0.5	34.3±2.9	5.5±0.6	39.9±4.7
No nicotinamide	4	1.1±0.2	2.4±0.6*	18.1±3.5†	2.4±0.9*	19.7±4.0*

Values are means ± SE on n independent experiments. In each experiment, porcine NIC aggregates were prepared from 3 pancreases and cultured for nine days in the presence or absence of 10 mmol/l nicotinamide. Stimulation indices were calculated by dividing the amount of insulin released at high glucose (20 mmol/l) or high glucose plus 10 mmol/l theophylline by that released at low glucose (2.8 mmol/l). Statistical significance of differences was calculated with unpaired Student's t-test (2-tailed).

*$p<0.05$, †$p<0.01$ vs. Ham's F10 plus 10 mmol/l nicotinamide.

Table IV. Composition of porcine neonatal islet cell grafts prior to transportation.

	Content (μg/graft)			Purity (μg/μg)	
Graft	Insulin	Glucagon	DNA	Insulin/DNA	Glucagon/DNA
1000 NIC aggregates	1.9±0.3	0.62±0.05	8.9±0.7	0.213±0.022	0.073±0.008
2000 NIC aggregates	4.0±0.4	1.32±0.11	18.9±1.5	0.219±0.025	0.072±0.007

Values are means ± SE of 10 independent experiments. In each experiment, porcine NIC aggregates were prepared from 3 pancreases.

Table V. Metabolic follow-up of porcine neonatal islet cell aggregate recipients.

Experimental Groups	n	Weeks Posttransplantation 2	4	6	8	10	12	14
		Normoglycemic Animals*/Total number of recipients						
Recipients								
1000 NIC's	13	0/13	2/13	5/13	8/13	10/13	10/13	10/13
2000 NIC's	20	4/20	6/20	13/20	20/20	20/20	20/20	20/20
		Mean Blood Glucose (mmol/l)						
Recipients								
1000 NIC's	13	23.8±0.6"	20.5±1.6"	14.8±1.7§	11.8±1.5‡	9.5±1.3	8.3±1.1	7.0±0.9
1000 NIC's†	10	23.7±0.5"	19.5±1.9"	12.7±1.6	9.5±1.3	7.0±0.2	6.3±0.3	5.3±0.2
2000 NIC's	20	18.7±1.5"	14.4±1.3‡	9.1±0.8	7.1±0.3	4.9±0.2	4.1±0.1‡	4.2±0.1§
Normal Controls	9	6.8±0.3	6.9±0.3	7.1±0.6	7.1±0.3	7.3±0.3	6.7±0.3	6.9±0.2

Values are means ± SE of n recipients.

*Normoglycemia defined as blood glucose values ≤ 8.4 mmol/l.

†Data represents means of only those recipients obtaining normoglycemia. Statistical significance of differences was calculated by one-way analysis of variance.

‡p<0.05, §p<0.01, "p<0.0001 vs. normal control group.

Table VI. Hormone content after transplantation of porcine NIC aggregates.

Experimental Group	n	Hormone content (μg/organ) Insulin	Glucagon
Normal Controls			
pancreas	9	36.5±1.3	0.53±0.02
Recipients of 1000 NIC aggregates			
pancreas	8	<0.7	0.51±0.03
kidney	8	63.7±9.2*	0.40±0.07
Recipients of 2000 NIC aggregates			
pancreas	14	<0.7	0.46±0.06
kidney	14	88.0±5.6†‡	0.47±0.05

Values are means ± SE for n animals. All recipients exhibited normal glycemia and were analyzed at 14 weeks posttransplantation. Statistical significance of differences was calculated by one-way analysis of variance.
*$p < 0.05$, †$p < 0.0001$ vs. normal control pancreases.
‡$p < 0.05$ vs. kidney grafts in recipients of 1000 NIC aggregates.

Table VII. Metabolic follow-up of microencapsulated porcine neonatal islet cell aggregate recipients.

Experimental Groups	n	Weeks Posttransplantation 1	2	3	4	5	6	7
		Normoglycemic Animals[a]/Total number of recipients						
Encapsulated								
Expt. No.								
1	1	4/4	4/4	3/4[b]	3/4	3/4	3/4	3/4
2	2	½	2/2	2/2	2/2	2/2	2/2	
3	3	2/3	3/3	3/3	3/3	ongoing		
4	2	2/2	2/2	ongoing				
Non-Encapsulated[c]								
Expt. No.								
1	3	0/3	0/3	1/3	1/3	2/3	2/3	3/3
2	4	0/4	0/4	0/4	1/4	1/4	2/4	
3	2	0/2	0/2	0/2	½	ongoing		
4	4	0/4	0/4	ongoing				

[a]Normoglycemia declined as blood glucose value ≤8.4 mmol/l.

[b]One recipient died while normoglycemic.

[c]Aggregates were cultured for 16 days in serum free HAM's F10. When non-encapsulated NIC aggregates were initially cultured for 8 days without serum then subsequently cultured for an additional 8 days in HAM's F10 containing 5% neonatal pig serum, the aggregates clumped together becoming necrotic and were thus not of sufficient quality for transplantation.

Table VIII. Metabolic follow-up of diabetic nude mice transplanted with 2000 neonatal porcine islets.

Experimental Groups	Site	Weeks Posttransplantation 1	2	3	4	5	6	8	42
		Normoglycemic Animals[a]/Total number of recipients							
No Serum									
non-encapsulated	k.c.	0/8	0/8	2/8	4/8	6/8	7/8	8/8	8/8
encapsulated	i.p.	0/10	0/10	3/10	5/10	8/10	10/10	10/10	10/10
Plus Serum									
encapsulated	i.p.	5/12	12/12	11/12	12/12	12/12	12/12	12/12	12/12
dissolved capsules	k.c.	2/9	9/9	9/9	9/9	9/9	9/9	9/9	9/9

k.c., kidney capsule; i.p., intraperitoneally.

[a]Normoglycemia defined as blood glucose values ≤8.4 mMl.

Table VIII. Metabolic follow-up of diabetic nude mice transplanted with 2000 neonatal porcine islets.

Experimental Groups	Site	Weeks Posttransplantation 1	2	3	4	5	6	8	42
		Normoglycemic Animals[a]/Total number of recipients							
No Serum									
non-encapsulated	k.c.	0/8	0/8	2/8	4/8	6/8	7/8	8/8	8/8
encapsulated	i.p.	0/10	0/10	3/10	5/10	8/10	10/10	10/10	10/10
Plus Serum									
encapsulated	i.p.	5/12	12/12	11/12	12/12	12/12	12/12	12/12	12/12
dissolved capsules	k.c.	2/9	9/9	9/9	9/9	9/9	9/9	9/9	9/9

k.c., kidney capsule; i.p., intraperitoneally.

[a]Normoglycemia defined as blood glucose values ≤8.4 mMl.

Table IX. Survival of Lewis rat islet allografts coencapsulated with Lewis Sertoli cells transplanted IP into diabetic Wistar-Furth recipients.

				Time Until Diabetes Recurrence (days)
Alginate	Sertoli Cells (10^3)/Islet	n	Individual	
	Mean±SEM			
MXG	None	15	8x3, 9x2, 10, 11x4, 12x2, 13, 14x2	
	10.4±0.6			
MXG	5.5±0.2	5	74, 76, 82, 95, 98	
	85±4.9			

SUMMARY

The present data indicate that viable porcine neonatal islet cells can be successfully isolated in large numbers by culturing collagenase digested pancreas for 9 days. Since we and others (34) observed that the endocrine portion of the neonatal pig pancreas does not contain intact and mature islets, but rather exhibits a random distribution of endocrine cells, no attempts were made to process these organs using methods conventionally used for isolating adult mammalian islets. Neonatal pig pancreases were therefore, digested according to a modification of the method of Korsgren et al (10) for preparing fetal pig islet cell clusters. Tissue culture was then used to enrich the preparations in endocrine cells prior to assessing their viability through an *in vitro* stimulation assay and by transplantation into diabetic nude mice. Selection of the media supplements was based on the following considerations: 1) serum was omitted due to its ability to promote the survival of contaminating fibroblasts and pancreatic exocrine cells, and based on previous reports (35,36), albumin was selected as a serum substitute; 2) IBMX was used for its capacity to stimulate DNA synthesis in rat islets (37), and its potential to enhance survival of cultured rat β cells (36); 3) 10 mmol/l glucose was added to enhance β cell replication (38, 39) and for its cytoprotective effect during culture of purified rat β cells (36); and 4) nicotinamide was included due to its ability to stimulate islet cell DNA replication (40), and its beneficial effect on the metabolic function of porcine fetal islet cells (12).

Using insulin content as a parameter, approximately 65% of the initial pancreatic β cell mass was recovered after the collagenase digestion phase. This index of β cell recovery is likely an under estimation because it does not take into account possible degranulation which could occur during the digestion. A nine day culture of the digest succeeded in eliminating the majority of contaminating exocrine cells and resulted in the formation of numerous islet cell aggregates. The NIC aggregates were predominantly composed of epithelial cells, exhibited a purity of 35% endocrine cells, and contained approximately 25% insulin-positive cells. With the technique used in this study, generally 50,000 NIC aggregates were recovered from one pancreas. These yields are significantly higher than those obtained in studies using mid-gestational fetal pigs, where the average yield per fetus was approximately 10,000 islet cell clusters (10,13). When considering the mean DNA recovery per pancreas, the DNA content of porcine neonatal islet cells (7.1 pg/cell),

and the percentage of insulin-positive cells, the following equation can be used to calculate total β-cell mass in the isolated NIC aggregates:

$$\frac{\text{Total DNA content}}{\text{7.1 pg DNA/NIC}} \times \frac{\text{\% insulin positive cells}}{100}$$

=number of β-cells recovered per pancreas

The number of β cells recovered from freshly isolated, 3-day, and 9-day cultured preparations is calculated to be 29.2, 8.5, 13.3 million cells per pancreas, respectively. Similarly, when considering the percentage of α-cells the calculated number of alpha cells is 11.7, 3.2, and 4.4 million cells per pancreas, respectively. The decrease in endocrine cell mass between the isolation and 3 days culture is likely the result of deleterious effects of the collagenase digestion and the presence of potentially cytotoxic proteases released from degenerating exocrine cells during culture. In contrast, the increase in both α and β cell mass (*i.e.* 38 and 56%, respectively) between day 3 and day 9 of culture, can possibly be explained by the growth or differentiation of new endocrine cells. Cellular insulin content also decreased significantly during the first 3 days of culture, and this is presumably related to the fall in β cell mass. However, between days 3 and 9 of culture, cellular insulin content decreased by 10%, yet β cell mass was shown to increase. Thus, even though new β cells were forming and contributing to the total insulin pool, their insulin stores as well as that of pre-existing β cells likely decreased as a result of secretory activity that exceeds the rate of insulin biosynthesis during culture. The recovery of cellular DNA decreased throughout the 9-day culture period, which is likely the result of the marked elimination of contaminating exocrine cells.

Many studies have indicated that the fetal β cell has a poor insulin response to glucose, which is rapidly converted to a more adult pattern after birth (22-25). In the present study, porcine neonatal β cells were capable of secreting significant amounts of insulin in response to a glucose challenge. This secretory capacity was further augmented when the NIC aggregates were pre-cultured in the presence of nicotinamide. These results are comparable to those described for islet cells prepared from the neonatal pig pancreas (1-3

days old) after trypsin dissociation and 7 days culture (27), and are significantly higher than those observed for fetal pig islet cells (10, 12); suggesting that in the pig, neonatal β cells are more responsive to glucose than fetal β cells. While not wanting to be bound by a particular theory, a possible explanation for nicotinamide's beneficial effect is that it may act as a protective agent, thereby preserving the insulin secretory activity of the neonatal β cell throughout the culture period. Nicotinamide has been shown to protect against the cytotoxic action of streptozotocin on β cells (41, 42) and to slow or arrest the development of diabetes in the non-obese diabetic mice (43). The agent may therefore enhance survival of porcine NICs after collagenase digestion and protect them from chemical disruption due to the proteolytic activity of enzymes leaking from degenerating exocrine cells during culture. Interestingly, culture in nicotinamide did not increase the frequency of insulin-positive cells in the NIC aggregates. This observation is in contrast to that observed when fetal porcine (12) and human (44) islet cell clusters were cultured with nicotinamide.

Diabetic nude mice were transplanted with 1000 or 2000 NIC aggregates, containing either 3 or 6 x 10^5 β cells. At posttransplantation week 14, 75% of the mice receiving 1000 aggregates exhibited blood glucose values ±8.4 mmol/l, whereas, 2000 aggregates cured diabetes in 100% of the animals within 8 weeks after implantation. In contrast, Davilli and associates demonstrated that 2000 adult porcine islet equivalents infrequently produced normoglycemia in diabetic nude mice, and that 4000 islet equivalents with an average cellular insulin mass of 38.4 ug required >5 weeks to normalize diabetic nude mice (15). In the present study, only 2000 porcine NIC aggregates containing 4 ug insulin, was required to achieve euglycemia long term. One possible explanation for these differences is that adult porcine islets are considerably more fragile than the NIC aggregates and this may result in significantly more β cell death and malfunction of the adult islets posttransplantation. It is also conceivable that during the initial days after implantation, reduction in the grafts β cell mass may occur due to poor vascularization and anoxic conditions. Thus, with adult islet grafts the final β cell mass that becomes functionally active following the engraftment period may be considerably less than the amount initially implanted. On the other hand, the ability of immature NICs to differentiate and proliferate may potentially allow them to replace any β cell mass lost to ischemic damage in the immediate posttransplantation period.

Although the NIC grafts grown in the absence of culture in microcapsules were unable to correct diabetes immediately after transplantation, they eventually developed the capacity to establish and maintain euglycemia, likely because the relatively few β-cells implanted initially were subsequently supplemented by the growth and/or differentiation of additional new β cells. Interestingly, all recipients survived this hyperglycemic period, yet diabetic controls survived for only 11±4 days after alloxan treatment, suggesting that even in the first two weeks posttransplantation, NIC grafts produced sufficient insulin to keep recipients alive, although not euglycemia. We hypothesize that a hyperglycemic environment may be essential to inducing the growth and differentiation of new β-cells in our experimental model, and perhaps also in the clinical setting. At the time of implantation, the insulin content of NIC grafts corresponded to only 5 - 10% of the pancreatic insulin content found in aged matched normal controls. Following transplantation, the grafts insulin mass increased by >20-fold. Whether this increase in insulin mass was related to the birth of new β cells through differentiation of epithelial cells in the NIC aggregates and/or replication of existing β cells was not assessed in this study. It has however, been suggested that the major source of newly formed β cells in transplanted porcine islet cell clusters is from undifferentiated epithelial cells rather than from pre-existing β cells (11). Our morphological data indirectly support this concept, since at the time of implantation NIC grafts were composed predominately of epithelial cells, whereas several weeks after transplantation, few epithelial cells were detected and insulin-producing β cells now comprised the major volume of the graft. It cannot be excluded, however, that β cell proliferation did not contribute to at least some of the increased insulin content of the grafts, as electron micrographs indicated mitotic activity within some of the engrafted β cells. The use of semi-quantitative morphometric techniques, such as bromodeoxyuridine labeling and simultaneous immunostaining for islet hormones (15), should provide further insight into the growth kinetics of porcine NIC grafts. It is worthy to note that the grafts glucagon content did not increase following transplantation, suggesting that no additional growth of a-cells occurred. Similarly, in studies where fetal porcine islet cells were implanted into alloxan-diabetic nude mice, the frequency of glucagon containing cells markedly decreased after transplantation (11). These observations indicate that in immature islet cell grafts, continued growth and differentiation of endocrine non-β cells is limited, if not inhibited, when transplanted under the kidney capsule of alloxan-diabetic nude mice.

However, neonatal islet cells cultured in alginate microcapsules for approximately 1 week, likely contained increased β-cell mass as indicated by an 89% increase in cellular insulin content. Therefore, once transplanted into the recipient, animals became normoglycemic in one to seven days. It is interesting to note that once normoglycemia is achieved, it appears that the transplanted cells no longer proliferate.

The pancreas of normal control mice was shown to contain about 40 ug insulin and grafts obtained from recipients of 2000 NIC aggregates contained 88 ug insulin. The apparent excess β cell mass in these animals could explain their lower blood glucose levels when compared to age-matched normal controls. On the other hand, this phenomenon has also been observed in nude mice transplanted with adult porcine islets in which the grafts β cell mass did not differ from that found in the pancreas of normal control mice (15). Thus, an alternative explanation for this observation is that the donors β cells eventually regulated the recipient's glucose homeostasis to that found in pigs, rather than in mice. Therefore, since plasma glucose levels in pigs and humans are similar, these results indicate that transplantation of porcine NICs into humans should theoretically maintain blood sugars within the recipients physiological range.

In conclusion, the neonatal porcine pancreas can be used for the isolation of a large number of functionally viable islet cells. Furthermore, due to their ready availability and inherent capacity to proliferate and differentiate both *in vitro* and *in vivo*, they constitute an attractive source of insulin-producing tissue for studies of islet cell neogenesis or as a source of xenogeneic islet cells for clinical transplantation.

The foregoing description of the invention is exemplary for purposes of illustration and explanation. It should be understood that various modifications can be made without departing from the spirit and scope of the invention. Accordingly, the following claims are intended to be interpreted to embrace all such modifications.

REFERENCES

1.Scharp, D.W., P.E. Lacy, J.V. Santiago, C.S. McCullough, L.G. Weide, L. Falqui, P. Marchetti, R.L. Gingerich, A.S. Jaffe, P.E. Cryer, C.B. Anderson, and M.W. Flye. 1990. Insulin independence after islet transplantation into type 1 diabetic patient. Diabetes 39:515-518.

2.Warnock, G.L., N.M. Kneteman, E. Ryan, R.E.A. Seelis, A. Rabinovitch, and R.V. Rajotte. 1991. Normoglycemia after transplantation of freshly isolated and cryopreserved pancreatic islets in type 1 (insulin-dependent) diabetes mellitus. Diabetologia 34:55-58.

3. Socci, C., L. Falqui, A.M. Davalli, C. Ricordi, S. Braghi, F. Bertuzzi, P. Maffi, A. Secchi, F. Gavazzi, M. Freschi, P. Magistretti, S. Socci, A. Vignali, V. Di Carlo, and G. Pozza. 1991. Fresh human islet transplantation to replace pancreatic endocrine function in type 1 diabetic patients. Acta Diabetol 28:151-157.

4. Warnock, G.L., N.M. Kneteman, E. Ryan, A. Rabinovitch, and R.V. Rajotte. 1992. Long-term follow-up after transplantation of insulin-producing pancreatic islets into patients with type 1 (insulin-dependent) diabetes mellitus. Diabetologia 35:89-95.

5. Ricordi, C., A.G. Tzakis, P.B. Carroll, Y. Zeng, H.L. Rodriguez Rilo, R. Alejandro, R. Shapiro, J.J. Fung, A.J. Demetris, D.H. Mintz, and T.E. Starzl. 1992. Human islet isolation and allotransplantation in 22 consecutive cases. Transplantation 53:407-414.

6. Alejandro, R., S. Strasser, V. Esquenazi, G. Burke, J. Nevry, J. Miller, E.T. Shapiro, C. Ricordi, and D. Mintz . 1994. Long-term survival of intraportal islet allografts in type 1 diabetes mellitus. In Pancreatic Islet Cell Transplantation. C. Ricordi editor. RG Landes Co., Austin. 410-413.

7. Gray, D.W.R., P. McShane, A. Grant, and P.J. Morris. 1984. A method for isolation of islets of Langerhans from the human pancreas. Diabetes 33:1055-1061.

8. Warnock, G.L., D.K. Ellis, R.V. Rajotte, I. Dawidson, S. Baekkeskov, and J. Egebjerg. Studies of the isolation and viability of human islets of Langerhans. 1988. Transplantation 45:957-963.

9. Ricordi, C., P.E. Lacy, E.H. Finke, B.J. Olack, and D.W. Scharp. 1988. Automated method for isolation of human pancreatic islets. Diabetes 37:413-420.

10. Korsgren, O., S. Sandler, A.S. LandstrÜm, L. Jansson, and A. Andersson. 1988. Large-scale production of fetal porcine pancreatic isletlike cell clusters. An experimental tool for studies of islet cell differentiation and xenotransplantation. Transplantation 45:509-514.

11. Korsgren, O., L. Jansson, D. Eizirik, and A. Andersson. 1991. Functional and morphological differentiation of fetal porcine islet-like clusters after transplantation into nude mice. Diabetologia 34:379-386.

12. Korsgren, O., A. Andersson, and S. Sandler. 1993. Pretreatment of fetal porcine pancreas in culture with nicotinamide accelerates reversal of diabetes after transplantation to nude mice. Surgery 113:205-214.

13. Groth, C.G., O. Korsgren, A. Tibell, J. Tollemar, E. MÜller. J. Bolinder. J. Ostman. F.P. Reinholt. C. HellerstrÜm, and A. Andersson. 1994. Transplantation of porcine fetal pancreas to diabetic patients. Lancet 344:1402-1404.

14. Liu, X., K.F. Federlin, R.G. Bretzel, B.J. Hering, and M.D. Brendal. 1991. Persistent reversal of diabetes by transplantation of fetal pig proislets into nude mice. Diabetes 40:858-866.

15. Davalli, A.M., Y. Ogawa, L. Scaglia, Y. Wu, J. Hollister, S. Bonner-Weir, and G.C. Weir. 1995. Function, mass. and replication of porcine and rat islets transplanted into diabetic nude mice. Diabetes 44:104-111.

16. Marchetti, P., R. Giannarelli, S. Cosimi, P. Masiello, A. Coppelli, P. Viacava. and R. Navalesi. 1995. Massive isolation, morphological and functional characterization. and xenotrans plantation of bovine pancreatic islets. Diabetes 44:375-381.

17. Ricordi, C., C. Socci, A.M. Davalli, C. Staudacher, P. Baro, A. Vertova, I. Sassi, F. Gavazzi, G. Pozza, and V. Di Carlo. 1989. Isolation of the elusive pig islet. Surgery 107:688-694.

18. Socci, C., C. Ricordi, A.M. Davalli, C. Staudacher, P. Baro, A. Vertova, M. Freschi, F. Gavazzi, S. Braghi, G. Pozza, and V. Di Carlo. 1989. Selection of donors significantly improves pig islet isolation yield. Horm Metab Res 25 (Suppl. 1):32-35.

19. Kirchhof, N., B.J. Hering, V. Geiss, K. Federlin, and R.G. Bretzel. 1994. Evidence for breed-dependent differences in porcine islets of Langerhans. Transplant Proc 26:616-617.

20. Van Deijnen, J.H.M., C.E. Hulstaert, G.H.J. Wolters, and R. van Schilfgaarde. 1992. Significance of the peri-insulinar extracellular matrix for islet isolation from the pancreas of the rat, dog, pig, and man. Cell Tissue Res 267:139-146.

21. Marchetti, P., E.H. Finke, C. Swanson, A. Gerasimidi-Vazeou, D.W. Scharp, R. Navalesi, and P.E. Lacy. 1992. The potential of porcine islet xenotransplantation in the therapy of diabetes. Diab Nutr Metab 5 (Suppl. 1):151-154.

22. Asplund, K., S. Westman, and C. HellerstrÜm. 1969. Glucose stimulation of insulin secretion from the isolated pancreas of foetal and new born rats. Diabetologia 5-:260-262.

23. Asplund G.. Dynamics of insulin release from the foetal and neonatal rat pancreas. 1973. Europ. J. Clin. Invest. 3:338-344.

24. Rhoten W.B.. 1980. Insulin secretory dynamics during development of rat pancreas. Am J Physiol 239:E57-E63.

25. Hole, R.L., M.C.M. Pian-Smith, and G.W.G. Sharp. 1988. Development of the biphasic response to glucose in fetal and neonatal rat pancreas. Am J Physiol 254:E167-E174.

26. Tuch, B.E., A. Jones, and J.R. Turtle. 1985. Maturation of the response of human fetal pancreatic explants. Diabetologia 28:28-31.

27. Korbutt, G.S., and D.G. Pipeleers. 1992. Cold storage of the rat pancreas before purification of islet β-cells. Diabetes 41:299-307.

28. Pipeleers, D.G., P.A. In't Veld, M. Van De Winkel, E. Maes, F.C. Schuit, and W. Gepts. 1985. A new *in vitro* model for the study of pancreatic A and B cells. Endocrinology 117:806-816.

29. Pipeleers, D.G., and M.A. Pipeleers-Marichal. 1981. A method for the purification of single A, B and D cells and for the isolation of coupled cells from isolated rat islets. Diabetologia 20:654-663.

30. Pipeleers, D.G., M. Pipeleers-Marichal, J.-C. Hannaert, M. Berghmans, P.A. In't Veld, J. Rozing, M. Van De Winkel, and W. Gepts. 1991. Transplantation of purified islet cells in diabetic rats. I. Standardization of islet cell grafts. Diabetes 40:908-919.

31. Korbutt, G.S., and Pipeleers D.G.. 1993. Rat pancreas preparation for cold storage and subsequent islet cell isolation. Transplantation 56:500-503.

32. Pipeleers D.G.. 1992. Composition of islet grafts. Diabetes Nutrition and Metabolism 5 (Suppl. 1):29-32.

33. Ricordi C.. 1991. Quantitative and qualitative standards for islet isolation assessment in humans and large mammals. Pancreas 6:242-244.

34. Alumets, J., R. Hakanson, and F. Sundler. 1983. Ontogeny of endocrine cells in porcine gut and pancreas. An immunocytochemical study. Gastroenterology 85:1359-1372.

35. Clark, S.A., and W.L. Chick. 1990. Islet cell culture in defined serum-free medium. Endocrinology 126:1895-1903.

36. Ling, Z., J.C. Hannaert, and D. Pipeleers. 1994. Effect of nutrients, hormones and serum on survival of rat islet beta cells in culture. Diabetologia 37:15-21.

37. Rabinovitch, A., B. Blondel, T. Murray, and D.H. Mintz. 1980. Cyclic adenosine-3',5'-monophosphate stimulates islet B cell replication in neonatal rat pancreatic monolayer cultures. J. Clin. Invest. 66:1065-1071.

38. Swenne, I., A.J. Bone, S.L. Howell, and C. HellerstrÜm. 1980. Effects of glucose and amino acids on the biosynthesis of DNA and insulin in fetal rat islets maintained in tissue culture. Diabetes 29:686-692.

39. HellerstrÜm, C., and I. Swenne. 1991. Functional maturation and proliferation of fetal pancreatic °-cells. Diabetes 40 (Suppl. 2):89-93.

40. Sandler, S., and A. Andersson. 1986. Long-term effects of exposure of pancreatic islets to nicotinamide *in vitro* on DNA synthesis. metabolism and B-cell function. Diabetologia 2 9:199-202.

41. Schein, P.S., D.A. Cooney, and M.L. Veron. 1967. The use of nicotinamide to modify the toxicity of streptozotocin diabetes without loss of antitumor activity. Cancer Res 27:2324-2332.

42. Sandler, S., M. Welsh, and A. Andersson. 1983. S treptozotocin-induced impairment of islet B-cell metabolism and its prevention by a hydroxyl radical scavenger and inhibitors of poly(ADP-ribose) synthetase. Acta Pharmacol Toxicol (Copenh). 53:392-400.

43. Yamada, K., K. Novaka, T. Hanafusa, T. Miyazaki. H. Toyoshima. and S. Tarui. 1987. Preventive and therapeutic effects of large-dose nicotinamide injections on diabetes associated with insulitus. An observation in nonobese diabetic (NOD) mice. Diabetes 31:749-753.

44. Otonkoski, T., G.M. Beattie, M.I. Mally, C. Ricordi, and A. Hayek. 1993. Nicotinamide is a potent inducer of endocrine differentiation in cultured human fetal pancreatic cells. J. Clin. Invest. 92:1459-1466.

All references and patent documents cited herein are incorporated by reference as if each were individually so denoted.

WHAT IS CLAIMED IS:

1. A method for transplantation of endocrine cells into a subject, wherein the subject would benefit from the transplantation, comprising:

a) digesting a tissue source of differentiated endocrine or endocrine precursor cells to form a digest;

b) culturing the digest in vivo in a serum-free basal media supplemented with a serum substitute, an agent to stimulate DNA synthesis, and an agent to promote endrocrine cell survival, resulting in an enriched population of endocrine cells; and

c) transplanting the cells into the subject.

2. A method for transplantation of hepatocytes into a subject, wherein the subject would benefit from the transplantation, comprising:

a) digesting a tissue source of liver cells to form a digest;

b) culturing the digest in vivo in a serum-free basal media supplemented with a serum substitute, an agent to stimulate DNA synthesis, and an agent to promote hepatocyte cell survival, resulting in an enriched population of hepatocytes; and

c) transplanting the hepatocytes into the subject.

3. A method for transplantation of insulin-secreting neonatal islet cells into a diabetic subject comprising:

a) digesting a pancreas with collagenase to form a digest;

b) culturing the digest in vitro in a serum-free basal media supplemented with a serum substitute, an agent to stimulate DNA synthesis, and an agent to promote endocrine cell survival, resulting in an enriched population of insulin-secreting neonatal islet cells; and

c) transplanting the cells into the subject.

4. The method of Claim 3 wherein the islet cells are transfected with an exogenous foreign gene prior to transplantation.

5. The method of Claim 3 wherein the neonatal islet cells are porcine or human.

6. The method of Claim 3 further comprising administering an immunosuppressive agent pre-transplantation, concurrent with transplantation, or post-transplantation.

7. The method of Claim 3 wherein the neonatal islet cells are microencapsulated.

8. An in vitro method for increasing the rate of maturation of an undifferentiated cell comprising encapsulating the cell within a stabilizing matrix or microcapsule to form an encapsulated or a microencapsulated cell and culturing the encapsulated or microencapsulated cell in vitro.

9. The method of Claim 8 wherein the microcapsule comprises alginate.

10. The method of Claim 8 wherein the cell is a porcine or human neonatal islet cell.

11. The method of Claim 8 wherein the microencapsulated cell is cultured in a serum-containing or serum-free media.

12. The method of Claim 8 further comprising co-encapsulating at least two cell types.

13. The method of Claim 12 wherein the cells are Sertoli cells and neonatal islet cells.

14. An in vitro method for increasing the rate of proliferation of a cell comprising encapsulating the cell within a stabilizing matrix or microcapsule to form an encapsulated or a microencapsulated cell and culturing the encapsulated or microencapsulated cell in vitro.

15. The method of Claim 14 wherein the microcapsule comprises alginate.

16. The method of Claim 14 wherein the cell is a porcine or human neonatal islet cell.

17. The method of Claim 14 wherein the microencapsulated cell is cultured in a serum-containing or serum-free media.

18. The method of Claim 14 further comprising co-encapsulating at least two cell types.

19. The method of Claim 18 wherein the cells are Sertoli cells and neonatal islet cells.

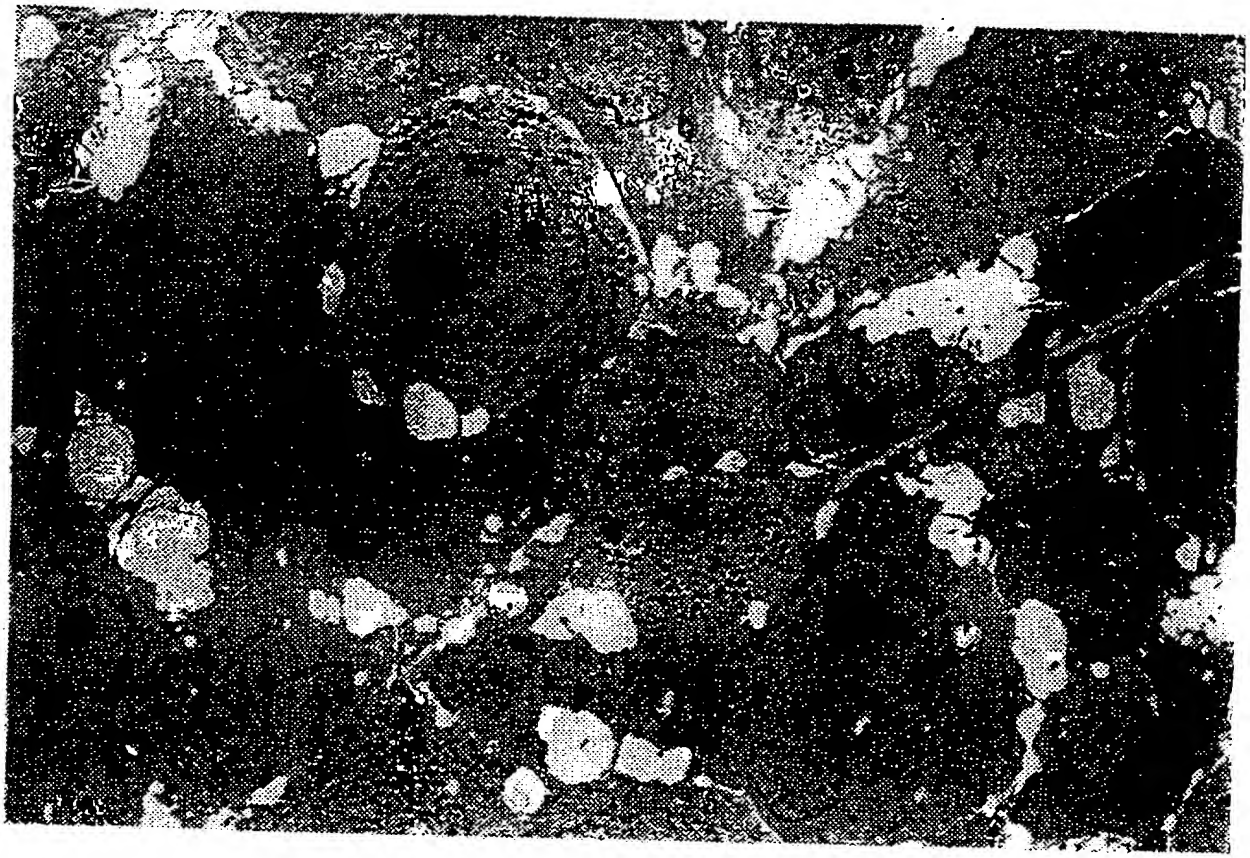

FIG. 1

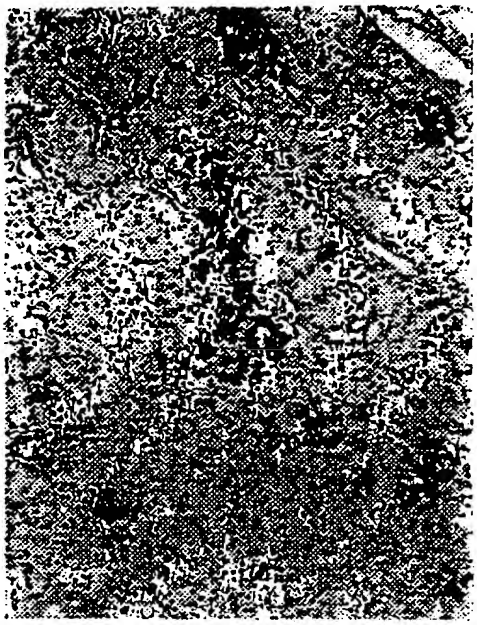

FIG. 2B

FIG. 2A

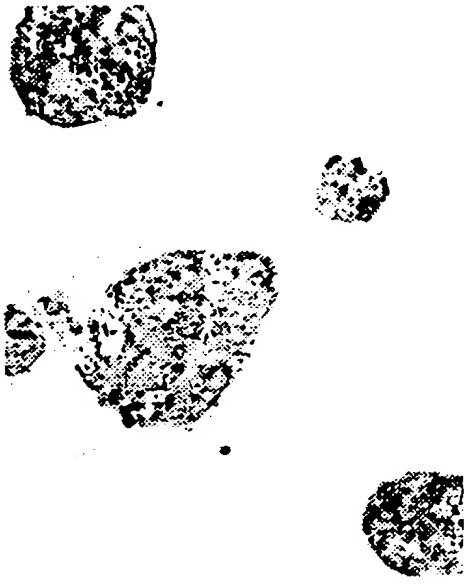

FIG. 2D

FIG. 2C

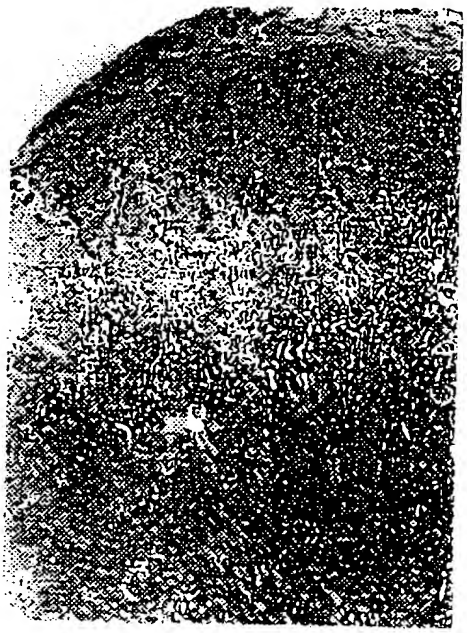

FIG. 2F

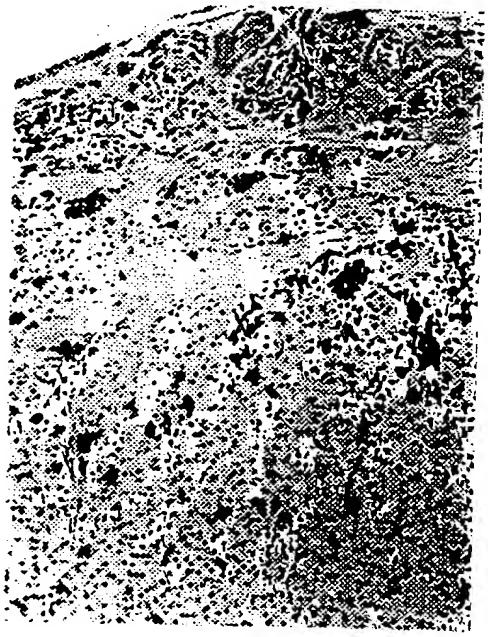

FIG. 2H

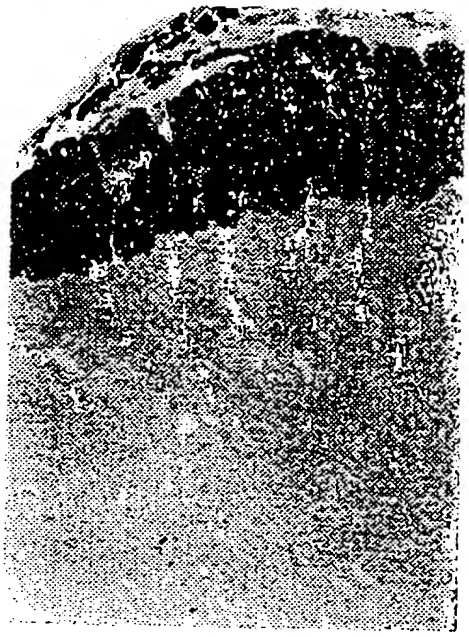

FIG. 2E

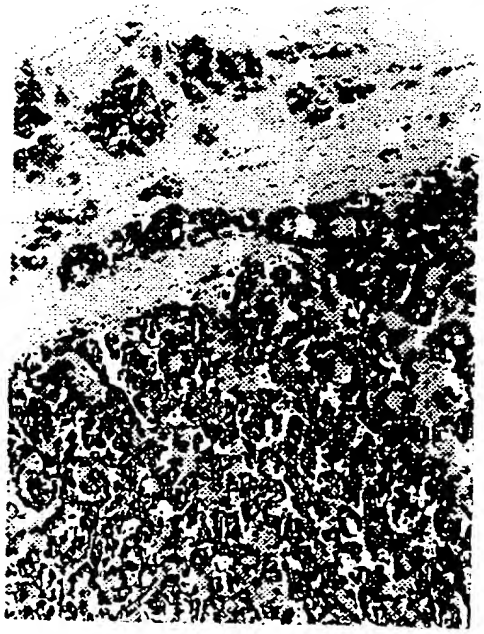

FIG. 2G

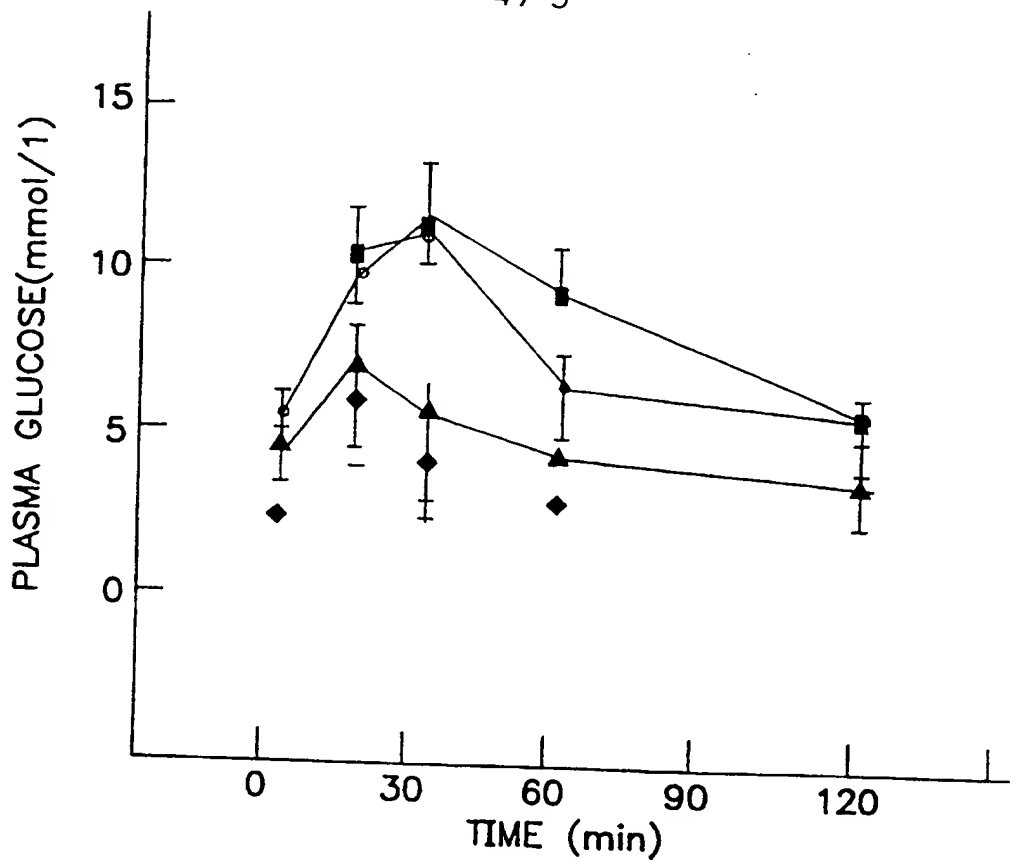
PLASMA GLUCOSE(mmol/1)
15
10
5
0
0
30
60
90
120
TIME (min)

FIG. 3A

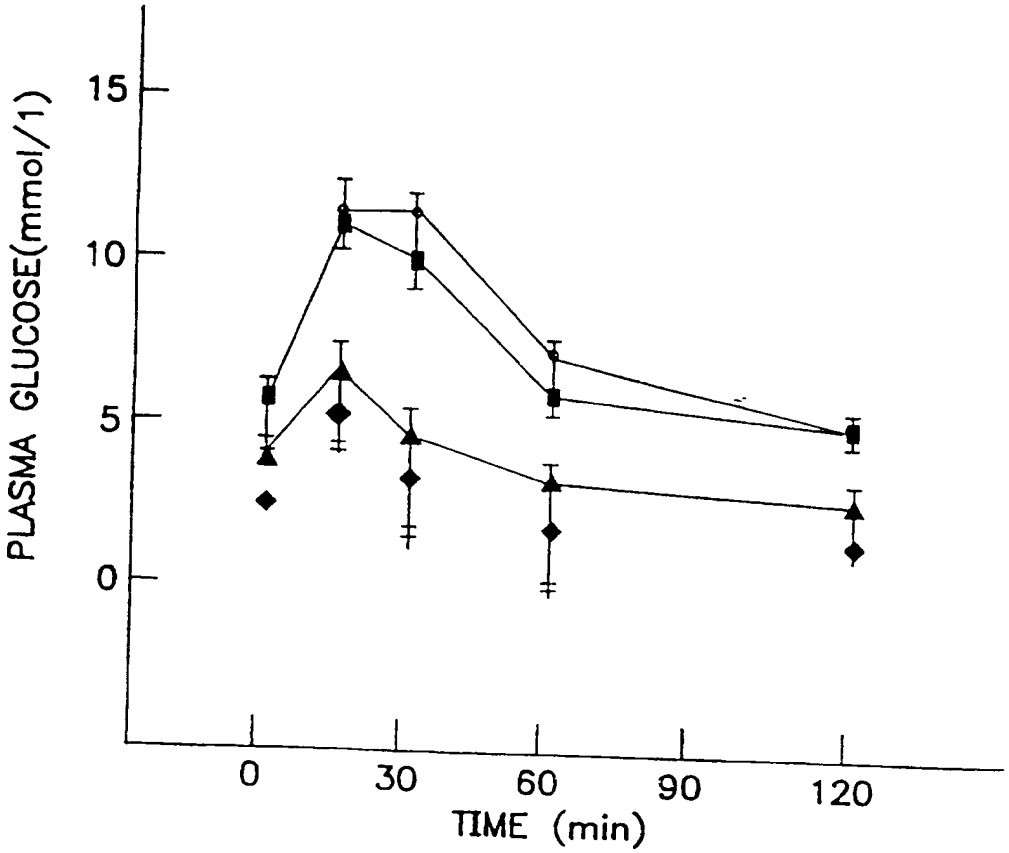
PLASMA GLUCOSE(mmol/1)
15
10
5
0
0
30
60
90
120
TIME (min)

FIG. 3B

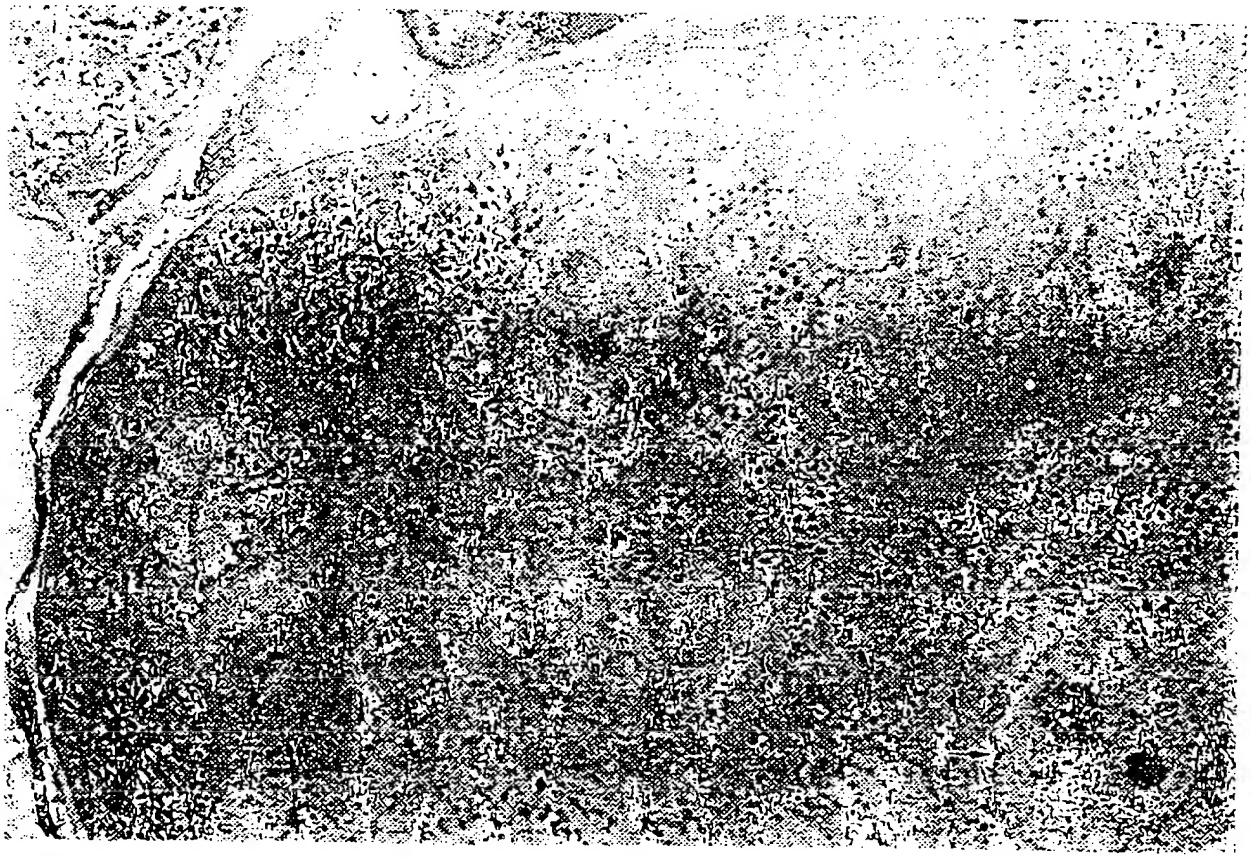

FIG. 4

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(74) Agent: MBM & CO.; 1000-100 Sparks Street, P.O. Box 809, Station B, Ottawa, Ontario K1P 5P9 (CA).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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(54) Title: METHODS FOR INCREASING THE MATURATION OF CELLS

(57) Abstract

The present invention provides a method of increasing the maturation rate or proliferation rate of a cell utilizing microencapsulation techniques. The invention also provides a method of treatment of a subject having diabetes utilizing cells produced by the culture method described herein.

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INTERNATIONAL SEARCH REPORT

Intern ial Application No
PCT/CA 97/00231

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/06 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 363 125 A (HANA BIOLOGICS INC.) 11 April 1990 see abstract see page 4, line 20 - page 14, line 21 ---	1,3,5
X Y	EP 0 127 989 A (CONNAUGHT LABORATORIES LTD) 12 December 1984 see abstract see the whole document & US 4 673 566 A (CONNAUGHT LAB.) 16 June 1987 cited in the application --- -/--	1-3,7-9, 11,14, 15,17 12,13, 18,19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
10 September 1997	28.10.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Panzica, G

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Inter nal Application No
PCT/CA 97/00231

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SELAWRY H.P. ET AL.: "Sertoli cell-induced effects on functional and structural characteristics of isolated neonatal porcine islets" CELL TRANSPLANTATION, vol. 5, no. 5, 1996, NEW YORK, pages 517-524, XP002040065 see the whole document	12,13, 18,19
X,P	KORBUTT G.S. ET AL.: "Large scale isolation, growth and function of porcine neonatal islet cells" JOURNAL OF CLINICAL INVESTIGATION, vol. 97, no. 9, 1 May 1996, NEW YORK, US, pages 2119-2129, XP002040062 see the whole document	1,3,5
X	WO 95 19743 A (UNIV. CALIFORNIA) 27 July 1995 see abstract see page 7, line 4 - page 9, line 18 see examples 3-5,8,9,11 see claims 32,33	1-3,7
A	US 5 227 298 A (WEBER COLLIN J. ET AL) 13 July 1993 cited in the application see abstract see column 11, line 5 - column 17, line 68	6-18
X,P A	WO 96 40178 A (RES CORP TECHNOLOGIES INC) 19 December 1996 see the whole document	1,3,6,7, 14,17-19 8,11-13
E	WO 97 16536 A (VIVORX INC.) 9 May 1997 see the whole document	1-3, 5-12, 14-18
A	WO 94 26915 A (UNIV MICHIGAN) 24 November 1994 see the whole document	4

INTERNATIONAL SEARCH REPORT

ational application No.
PCT/CA 97/00231

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2 ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3 ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1) Claims 1-7: method for the preparation of a tissue prior to a transplantation, namely its selection, digestion, culturing cells, their preparation and transplantation.
2) Claims 8-19: use of encapsulation to obtain some particular effects like increased maturation and proliferation rates.

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. ...nal Application No
PCT/CA 97/00231

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0363125 A	11-04-90	JP 2200178 A	08-08-90
EP 0127989 A	12-12-84	CA 1196862 A	19-11-85
		EP 0127713 A	12-12-84
		US 4673566 A	16-06-87
		JP 1428661 C	09-03-88
		JP 60258121 A	20-12-85
		JP 62038327 B	17-08-87
WO 9519743 A	27-07-95	US 5578314 A	26-11-96
		CA 2180737 A	27-07-95
		EP 0741550 A	13-11-96
		US 5643594 A	01-07-97
US 5227298 A	13-07-93	NONE	
WO 9640178 A	19-12-96	AU 6105696 A	30-12-96
WO 9716536 A	09-05-97	AU 7443996 A	22-05-97
WO 9426915 A	24-11-94	AU 6829994 A	12-12-94
		CA 2162602 A	24-11-94
		EP 0698109 A	28-02-96
		JP 8509869 T	22-10-96